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13. ABSTRACT *(Maximum 200 Words)*

The 2000 Alternative Toxicological Methods for the New Millennium: Science and Application symposium was held at the National Institutes of Health's National Library of Medicine from November 28 to December 1, 2000. The National Defense Authorization Act and National Institutes of Health Revitalization Act have directed DoD and NIH to establish aggressive programs to reduce, refine, or replace the use of research animals. This symposium gathered prominent international researchers to present the latest issues, research and trends toward addressing these needs. The symposium included platform presentations and poster sessions.

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Introduction

The National Defense Authorization Act and National Institutes of Health Revitalization Act have directed DoD and NIH to establish aggressive programs to reduce, refine, or replace the use of research animals. To address these needs, the Chief Toxicologist of the Edgewood Chemical Biological Center has organized and coordinated a series of biennial symposia. The 2000 Alternative Toxicological Methods for the New Millennium: Science and Application symposium was the sixth in the series, and was held at the National Institutes of Health's National Library of Medicine from November 28 to December 1, 2000. Several DoD and commercial organizations that are actively exploring alternative toxicological methods, including the US Army Medical Research Institute of Chemical Defense, supported the symposium. This symposium gathered prominent international researchers to present the latest issues, research and trends in alternatives to animal use in toxicological research via platform and poster presentations.

Body

Planning for the 2000 Alternative Toxicological Methods for the New Millennium: Science and Application symposium began in June 2000 with the selection of a steering committee and session chairs. The steering committee developed a draft agenda based on the outstanding issues in alternatives research. The dates of the symposium were then reserved with the National Library of Medicine at the National Institutes of Health (NIH) Bethesda campus. A meeting announcement was mailed to prospective attendees. The meeting was also advertised on Internet sites hosted by professional societies and sponsoring organizations.

The session chairs recruited renowned experts to present the latest research results and trends in their respective fields. Thus, all the platform presentations were invited speakers. To broaden the participation and topical areas, a poster session was also planned. With contractor assistance, a final agenda for the symposium, complete with abstracts of the presentations, was developed. A detailed final meeting announcement was mailed. The abstracts were then assembled into a Program Book, which was printed and distributed at the symposium. The text of the Program Book is included as Appendix A. Commitments were also solicited from the speakers to provide written manuscripts of their presentations, which would be included in the symposium proceedings. Monthly Progress Reports submitted by the contractor during the planning and conduct of the symposium are included as Appendix B.

The symposium was organized along the lines of a typical scientific meeting. The symposium began with early registration and two poster sessions, which were followed by a welcome reception hosted by an industry sponsor. The succeeding days were devoted to focused sessions of platform presentations. Appropriate time was set aside for open discussion through question and answer. A dinner banquet, which was included in the registration fee for the symposium, was held mid-week. It featured a dinner speaker and the presentation of awards to participants in the symposium.

Key Research Accomplishments

- The 2000 Alternatives symposium had over 150 attendees – one of the largest attendance figures to date.
- Speakers and poster presenters represented 8 military organizations, 9 government agencies and laboratories, 10 industries, and 14 academic centers.
- There were 37 talks presented over 3-1/2 days; 32 posters were presented.
- 5 speakers were from foreign countries.

Reportable Outcomes

The 2000 Alternatives symposium was, by every measure, highly successful in raising awareness, promoting dialogue, and advancing the field of alternatives to animal testing in toxicological research. The follow-on to this symposium will be a set of proceedings, which will be published either as a book or as a special issue of the Journal of Applied Toxicology. At the present, most of the manuscripts needed to publish the proceedings have been received. They are being edited and organized into a logical sequence that effectively captures the state-of-the-art findings presented at the symposium. A preliminary outline of the proceedings is provided at Appendix C.

Conclusions

The 2000 Alternatives symposium met its stated aims with the help of sponsoring organizations such as USAMRICD and MRMC. The latest trends in alternatives to animal testing in toxicology research were shared among DoD, industry, government agencies, and academia. The enthusiastic response to this symposium demonstrates the value of the continuation of this successful series of biennial symposia.

References

Published proceedings from previous Alternatives symposia include:

Toxicity Assessment Alternatives: Methods, Issues, Opportunities, Harry Salem (editor) and Sidney A. Katz (editor), Humana Press (1999).

Advances in Animal Alternatives for Safety and Efficacy Testing, Harry Salem (editor) and Sidney A. Katz (editor), Taylor & Francis (1997).

Animal Test Alternatives: Refinement, Reduction, Replacement, Harry Salem (editor), Marcel Dekker (1995).

Appendix A. Technical Program Book developed for the 2000 Alternative Toxicological Methods for the New Millennium symposium

Alternative Toxicological Methods for the New Millennium: Science and Application

November 28 to December 1
Lister Hill Center
National Library of Medicine
National Institutes of Health
Bethesda, Maryland

2000

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Introduction

Welcome to the 2000 Alternative Toxicological Methods for the New Millennium: Science and Application symposium. The National Defense Authorization Act and National Institutes of Health Revitalization Act have directed DoD and NIH to establish aggressive programs to reduce, refine, or replace the use of research animals. This conference will present the latest issues, research and trends toward addressing these needs.

Symposium Sponsors

This symposium is made possible by the generous support of the following organizations:

U.S. Army Soldier and Biological Chemical Command (SBCCOM)

National Institute of Environmental Health Sciences (NIEHS)

U.S. Army Medical Research Institute of Chemical Defense (MRICD)

U.S. Army Center for Health Promotion and Preventive Medicine (CHPPM)

The U.S. Navy

The U.S. Air Force

Xenogen Corporation

The Gillette Company

The Humane Society of the United States

DermTech International

Interagency Committee on Neurotoxicology (ICON)

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Capital Area Chapter – Society of Toxicology (NCAC-SOT)

Association of Government Toxicologists (AGT)

Program Committee

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Dr. Robert Weichbrod
The Jackson Laboratory

Dr. Neil Wilcox
The Gillette Company

Dr. Errol Zeiger
National Institute of Environmental
Health Sciences

Technical Agenda

Tuesday, November 28	
11:00 am	**Early Registration**, Lister Hill Center Lobby
Poster Session Lister Hill Center Lobby (Posters remain on display through Thursday) Chair: Laurie Roszell, Ph.D. (US Army CHPPM)	
1:00 - 2:30 pm	Session 1: Dermal and Analytical Assessment
3:00 - 4:30 pm	Session 2: Neurotoxicity , Mutagenicity and Carcinogenicity
5:00 - 8:00 pm	**Wine & Cheese Reception** Holiday Inn Bethesda

Wednesday, November 29	
7:30 - 8:30 am	**Continental Breakfast**
8:30 to 8:40	**Welcome** – Mr. Mike Parker, Deputy to the Commander, Soldier and Biological Chemical Command
8:40 to 9:05	**Keynote Address** – Anne Sassaman, Ph.D., Director, Division of Extramural Research and Training, NIEHS
Progress in the Validation and Regulatory Acceptance of Alternatives Co-Chairs: Neil Wilcox, D.V.M., M.P.H. (Gillette Company) and Bill Stokes, D.V.M. (NIEHS)	
9:05 - 9:15	**Introduction and Overview of Session** Neil Wilcox, D.V.M., M.P.H. (Gillette Company)
9:15 - 9:45	**Update on the ICCVAM** Bill Stokes, D.V.M. (NIEHS)
9:45 - 10:15	**Validation and Regulatory Acceptance of Alternative Test Methods: Current Situation in the European Union** Julia Fentem, Ph.D. (Unilever Research) and Michael Balls, Ph.D. (ECVAM)
10:15 - 10:30	**Break**
10:30 - 11:00	**Integrated In Vitro Approaches for Assessing Acute Toxicity** Anna Forsby, Ph.D. (Stockholm University)
11:00 - 11:30	**Validation and Regulatory Status of Alternative Methods to Replace the Conventional LD50 Test** Kathy Stitzel, Ph.D. (Procter & Gamble Company)
11:30 - 12:00	**The OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation** Errol Zeiger, Ph.D. (NIEHS)
12:00 - 1:00 pm	**Lunch**

Development of Predictive Methods Based on Mechanisms of Eye Irritation at the Ocular Surface: Meeting Industry/Regulatory Needs
Co-Chairs: Sherry Ward, Ph.D. (Gillette) and Wiley Chambers, M.D. (FDA)

1:00 - 1:05 pm	**Introduction & Symposium Overview and Goals** Sherry Ward, Ph.D. (Gillette Company)
1:05 - 1:20	**Industry Perspective – Meeting Industry Needs for Draize Alternatives** Leon Bruner, D.V.M. (Gillette Company)
1:20 - 1:50	**Pathophysiology and Clinical Features of Thermal and Chemical Eye Injuries** Martin Reim, M.D.
1:50 - 2:10	**Evaluation and Refinement of the Bovine Cornea Opacity and Permeability Assay** John Ubels, Ph.D. (Calvin College)
2:10 - 2:25	**The Use of Corneal Organ Culture as an Ex Vivo Model for Ocular Toxicity Test of Commercial Hair Care Products** Fu-Shin Yu, Ph.D. and Ke-Ping Xu, M.D. (Harvard University)
2:25 - 2:40	**Break**
2:40 - 2:55	**Extent of Corneal Injury as the Mechanistic Basis for the Development of Alternative Ocular Irritation Tests** James Jester, Ph.D. (Univ. of Texas Southwestern Medical Center) and James K. Maurer, D.V.M., Ph.D. (Procter & Gamble Company)
2:55 – 3:10	**Area and Depth of Injury in a Rabbit Ex Vivo Corneal Model** Rosemarie Osborne, Ph.D. (Procter & Gamble Company)
3:10 - 3:25	**Engineered Tissue Equivalents for Ocular Irritancy Screening** May Griffith, Ph.D. (Ottawa General Hospital)
3:25 - 3:40	**The EpiOcular Prediction Model: A Reproducible In Vitro Means of Assessing Ocular Irritancy** Patrick Hayden, Ph.D. (MatTek Laboratories)
3:40 - 3:55	**Cell Biology of Immortalized Human Corneal Epithelial Cells in 3-Dimensional Cultures for In Vitro Toxicology Assays** Roger Beuerman, Ph.D. (Louisiana State University Health Sciences Center), Doan Nguyen, Bart De Wever
3:55 - 4:10	**Performance of the HCE-T TEP Human Corneal Epithelial Transepithelial Fluorescein Permeability Assay** Sherry Ward, Ph.D. (Gillette Company)
4:10 - 4:25	**Regulatory Perspective – Meeting Regulatory Agency Needs for Draize Alternatives** Wiley Chambers, M.D. (FDA)
4:25 - 5:00	**Discussion / Questions**
6:00 - 8:30 pm	**Dinner Reception** Holiday Inn Bethesda Dinner Speaker: James MacGregor, Ph.D., D.A.B.T. FDA Center for Drug Evaluation and Research **The Biotechnology Revolution and the Evolution of Regulatory Toxicology**

Thursday, November 30

7:00 - 8:00 am	**Continental Breakfast**
Dermal Co-Chairs: Bill Smith, Ph.D. (MRICD), Larry Rheins, Ph.D. (DermTech International)	
8:00 - 8:30	**Validating In Vitro Dermal Absorption Studies: An Introductory Case Study** Bob Zendzian, Ph.D. (EPA), Michael Dellarco, Dr. PH (EPA)
8:30 - 9:00	**Utility of Gene Array Technology in Skin Biology** Brian Jones, Ph.D. (Avon)
9:00 - 9:30	**Keratinocyte-Based Mechanistic Assays for Chemicals that Cause Contact Dermatitis** Anthony Gaspari, M.D. (Dept. Dermatology, Univ. of Rochester)
9:30 - 10:00	**Break**
10:00 - 10:30	**A Molecular Diagnostic Approach to Irritant or Allergic Patch Testing Using the DermPatch** Melissa Fitzgerald, Ph.D., Lawrence A. Rheins, Ph.D., Vera Morhenn, M.D., Stacey Humphrey and Nirmala Jayakumar (DermTech International)
10:30 - 11:00	**Standardized Human Epidermal and Mucosal Tissue Models for the Screening of Potential Lead Compounds in Pharmaceutical Development** Bart De Wever, M.S. (Skinethic Laboratories)
11:00 - 11:30	**In Vitro Skin Equivalent Models for Toxicity Testing** Patrick Hayden, Ph.D. (MatTek Laboratories)
11:30 - 1:00	**Lunch**
Neurotoxicology (Molecular Biomarkers, Transgenics and Imaging Technologies) Interagency Committee on Neurotoxicology (ICON) Co-Chairs: Thomas Sobotka, Ph.D. (FDA), William Slikker, Jr., Ph.D. (FDA)	
1:00 - 1:30 pm	**Methamphetamine-induced Neurotoxicity: Lessons from Genetically Engineered Mice** Jean Lud Cadet, M.D. (NIDA)
1:30 - 2:00	**Development and Evaluation of in vitro Imaging Techniques Used to Screen Agents That Affect Neuronal Differentiation** Stanley Barone, Jr., Ph.D. (EPA)
2:00 - 2:20	**Break**
2:20 - 2:50	**Potential Applications of Noninvasive Imaging in Toxicology Research** David Lester, Ph.D. (FDA)
2:50 - 3:20	**Molecular Strategies for Neurotoxicity Screening: Moving Beyond the One Compound, One Mechanism Approach** James O'Callaghan, Ph.D. (CDC)

3:20 - 3:50	**Development of a Model of Integrin Expression as a Molecular Biomarker for Early, Sensitive Detection of Neurotoxicity** Joyce Royland, Ph.D. (EPA)
3:50 - 4:20	**Summation**

Friday, December 1

7:00 - 8:00 am	**Continental Breakfast**

Role of Transgenics and Toxicogenomics in the Development of Alternative Toxicity Tests
Co-Chairs: Jerry Heindel, Ph.D. (NIEHS) and John Frazier, Ph.D. (US Air Force)

8:00 - 8:05	**Introduction** Jerry Heindel, Ph.D. (NIEHS)
8:05 - 8:30	**The Use of Transgenics in Carcinogenicity Testing: An Update** Denise Robinson, Ph.D. (ILSI)
8:30 - 9:20	**Toxicogenomics and Toxicity Testing** Ben Van Houten, Ph.D. (NIEHS)
9:20 - 10:10	**Mammalian Gene-Trapping with the Beta-Lactamase Reporter – Application to Toxicogenomics** Gregor Zlokarnik, Ph.D. (Aurora Biosciences Corporation)
10:10 - 10:30	**Break**
10:30 - 11:10	**Noninvasive Real-time Toxicodynamics** Karen Steinmetz, Ph.D. (Xenogen)

Role of Imaging in the Development of Alternative Toxicity Tests
Co-chairs Doug Kawahara, Ph.D. (Xenogen) and Karen Hamernik, Ph.D. (EPA)

11:10 - 12:00	**Nanostructures and Toxicology** Martin Philbert, Ph.D. (University of Michigan)
12:00 - 1:00 pm	**Lunch**
1:00 - 1:50	**MRI and Biophotonic Imaging** Brian Ross, Ph.D. (University of Michigan)
1:50 - 2:40	**Two Photon Microscopy: Application to Toxicology** Peter So, Ph.D. (MIT)
2:40 - 3:00	**Concluding discussion and remarks** Doug Kawahara, Ph.D. (Xenogen)
3:00	**Meeting Summation** Harry Salem, Ph.D. (US Army SBCCOM)

Social Events

You are invited to attend a

Wine & Cheese Reception

Sponsored by the Gillette Company

Tuesday evening, November 28, from 5:00 to 8:00 pm at the Holiday Inn Bethesda, 8120 Wisconsin Avenue (about 2 blocks south of the National Library of Medicine Facility)

Vegetables, dips and chips
Cheese platter Wine
Soft drinks

Take this opportunity to mingle with other symposium attendees, renew acquaintances, share common interests and ideas, and make social plans.

Included in the Symposium Registration is a

Dinner Banquet

Wednesday evening, November 29, from 6:00 to 8:30 pm at the Holiday Inn Bethesda

Guest Speaker is

James MacGregor, Ph.D., D.A.B.T.

FDA Center for Drug Evaluation and Research

The Biotechnology Revolution and the Evolution of Regulatory Toxicology

Dinner includes your choice of
Entrees Salads
Vegetables Beverages
Desserts

Presentations

Tuesday, November 28

Poster Session

Session Chair: Laurie Roszell, Ph.D.
US Army Center for Health Promotion and Preventive Medicine

Session 1: Dermal and Analytical Assessment

Vesicant Medical Countermeasure Based on the Apoptosis or Programmed Cell Death Paradigm

R. Ray[1], K. R. Bhat[2], B. J. Benton[1], A. Sparks[1], D. R. Anderson[1], J. P. Petrali[1], W. J. Smith[1], P. Ray[3] and D. S. Rosenthal[4]
[1]US Army Medical Research Institute of Chemical Defense, APG, MD 21010,
[2]Lincoln University, Lincoln University, PA 19352
[3]Walter Reed Army Institute of Research, Washington, D. C. 20307 and
[4]Georgetown University School of Medicine, Washington, D.C. 20007

Mustard gas, which is the common name for the skin blistering chemical warfare agent sulfur mustard (bis-(2-chloroethyl) sulfide, HD), causes death of rapidly dividing basal epidermal keratinocytes resulting in dermal-epidermal separation and, therefore, vesication. At present, there is no effective medical countermeasure for this extremely debilitating chemical threat, which potentially can create a severe handicap for a fighting force. It has been reported (Dabrowska *et al.*, Toxicol. Appl. Pharmacol., **141**:568-583, 1996) that cultured human epidermal keratinocytes (HEK) exposed to ≤ 250 μM HD die exclusively via apoptosis, whereas HEK exposed to ≥ 500 μM HD die via both apoptosis and necrosis. It, therefore, appears that apoptosis is an initial event in the mechanism of cell death due to HD. We studied apoptosis in HEK exposed to HD and observed that HD-induced apoptosis can be regulated, i.e., either inhibited or stimulated by manipulating two enzymes, poly (ADP-ribose) polymerase (PARP) or DNA-dependent protein kinase (DNA-PK). This observation provides a basis for a prospective approach toward a pharmacological intervention to alleviate vesicant injury via regulation of apoptosis.

We reported that in cultured HEK, HD (300 μM) causes apoptosis and cytotoxicity (Rosenthal *et al.*, J. Invest. Dermatol., **111(1)**:64-71, 1998; Ray *et al.*, The FASEB J., **14(8)**:A1518, 2000). Apoptosis was indicated by the appearance of the apoptotic bodies (scanning electron microscopy), formation of the characteristic nucleosome-sized DNA ladders, TUNEL-positive staining of cells, activation of the cysteine protease caspase-3/apopain, and cleavage of the death substrate PARP. Cytotoxicity was evidenced biochemically by lactate dehydrogenase (LDH) release, protease stimulation, and loss of membrane integrity, as well as on a morphological basis by monitoring cellular degeneration (light microscopy). We also observed that both activated PARP and DNA ligase I are involved in HD-induced DNA damage repair (Bhat *et al.*, In Vitro Mol. Toxicol., **11(1)**: 45-53, 1998) and that this DNA ligase I activation is via DNA-PK mediated phosphorylation (Bhat *et al.*, The FASEB J., 13(7): A1453, 1999). Our results indicated that manipulating PARP activity and/or DNA repair could regulate DNA damage-induced apoptosis and cytotoxicity. In HEK, preventing PARP stimulation following HD by 10 mM benzamide (PARP inhibitor) delayed apoptosis in a time-dependent manner and, in parallel, attenuated cytotoxicity. On the contrary, interfering with the DNA repair pathway by inhibiting DNA ligase I activation via DNA-PK inhibition by dimethyl aminopurine (DMAP, 1 mM) accelerated apoptosis and enhanced the cytotoxic effects of HD. Since DMAP is not a specific DNA-PK inhibitor we confirmed this observation by using two cell lines, one containing and the other lacking DNA-PK activity. HD-induced apoptosis and cytotoxicity were enhanced in the DNA-PK deficient clonal cell line M059J compared with its DNA-PK containing sister clone, M059K. These results show that (a) apoptosis is an important cytotoxic response to HD exposure, and (b) HD-induced

apoptosis can be regulated by pharmacological means.

The 300 μM HD concentration used in our study is physiologically meaningful because this concentration is considered to be equivalent to a vesicating dose for human skin. To explain the significance of our observation that HD-induced apoptosis is delayed by PARP inhibition, but is accelerated by DNA repair inhibition we propose two opposing dynamics of cellular responses to 300 μM HD insult as follows. HD-induced DNA damage triggers two independent events: (a) PARP stimulation leading to apoptosis, and (b) activation of DNA repair opposing the progression of apoptosis. Under these circumstances, inhibition of DNA repair should tip the balance toward apoptosis, as supported by our results. Facilitating DNA repair should retard apoptosis. Based on this paradigm, inhibiting PARP and simultaneously promoting DNA repair appears to be a rational strategy for developing an effective vesicant medical countermeasure. A number of potential PARP inhibitors with demonstrated antivesicant efficacy both *in vitro* and *in vivo* have been identified through our in-house and extramural drug assessment programs. The results of our research on HD-damaged DNA repair described here have elucidated some key mechanisms that can be exploited to promote DNA repair. In conclusion, the concepts supported by our experimental results described here appear to be highly promising for development of a fielded antivesicant antidote, which is urgently needed for protecting our war fighters.

DNA Repair in Cultured Human Epidermal Keratinocytes Exposed to Sulfur Mustard

K. R. Bhat, B. J. Benton, A. Sparks and R. Ray
US Army Medical Research Institute of Chemical Defense, APG, MD 21010

One of the approaches in preventing cellular toxicity following sulfur mustard (SM) exposure is to facilitate DNA repair prior to attempted mitosis of the exposed cells. A clear understanding of the DNA repair mechanism in SM-exposed cells is therefore a prerequisite for this objective. A qualitative agarose gel analysis of DNA extracted from SM-exposed normal human epidermal keratinocytes (NHEK) showed a progressive increase in high molecular weight DNA, suggesting a time-dependent (0.5-4 hr) DNA repair. In SM-exposed cells, there was also a rapid (□1 hr) and dose-dependent (0.1-1 mM) activation (100% above unexposed control) of the 130 KDa DNA repair enzyme DNA ligase I, followed by a decay with a rate constant of □0.5 hr^{-1}. Along with DNA ligase I, another DNA repair enzyme, poly(ADP-ribose) polymerase (PARP), is known to be activated following DNA damage. The decay rate of activated DNA ligase I decreased by 60% in the presence of either a PARP inhibitor or an intracellular calcium chelator. In NHEK, inhibition of PARP also decreased the nuclease-induced TUNEL reaction. These results suggest that PARP is involved in the incision-excision and ligation steps of the DNA repair mechanism. We studied the mechanism of DNA ligase activation, assuming that it could be due to PARP-mediated ADP-ribosylation of this enzyme. Purified DNA ligase derived from SM-exposed NHEK in which NAD^+ was labeled with ^3H on adenine did not contain the ADP-ribose moieties. However, analysis of the cell extracts obtained from NHEK exposed to SM in the presence of $^{33}PO_4^{3-}$ indicated an association of the radiolabel with DNA ligase, a result consistent with the reported activation of DNA ligase via phosphorylation. The results of our studies utilizing the bovine DNA ligase I monoclonal antibody affinity chromatographic procedures confirm that DNA ligase I protein phosphorylation is the mechanism of its activation due to SM, and that this phosphorylation may be mediated by the DNA-dependent protein kinase, DNA-PK. These findings suggest that DNA ligase I and PARP activations may be two distinct events in DNA repair following alkylation. We propose a mechanism in which PARP molecules first bind to and stabilize DNA strand breaks to render the damaged sites more amenable to repair. Following the synthesis of the repair patch, PARP is released on self ADP-ribosylation due to reduced affinity. Activated DNA ligase I then completes the ligation to end the repair process.

Sulfur Mustard Stimulates the Transcription of a Serine Protease in Cultured Human Keratinocytes

P. Ray[1], F. G. Sheikh[1] and R. Ray[2]

[1]Walter Reed Army Institute of Research,
Washington, D.C. 20307 and
[2]US Army Medical Research Institute of
Chemical Defense,
Aberdeen Proving Ground, MD 21010

Sulfur mustard (SM, (bis-(2-chloroethyl) sulfide) causes skin blisters as a result of dermal-epidermal separation due in part to proteolytic degradation of some structural components responsible for the dermal-epidermal attachment. We purified a SM-stimulated ≅ 70 KDa serine protease, which is dependent on Ca^{2+} and new protein synthesis (cycloheximide sensitive). This protease hydrolyzes laminin which is a structural protein at the dermal-epidermal junction. We studied the mechanism of this protease stimulation by Northern blot analysis of SM-stimulated mRNA utilizing synthetic sense as well as antisense oiligodeoxynucleotides corresponding to amino acid sequences of two peptide fragments of this purified protease. Protease transcription was detectable in untreated keratinocytes (80-90 % confluent), and was optimally stimulated by SM at 25 μM. Transcription was blocked by either an irreversible (actinomycin D) or a reversible (DRB) transcription inhibitor, and also by the antisense oligos. These results may be useful in preventing SM injury via intervention at the level of protease transcription induced by SM.

Effects of Low Dose Sulfur Mustard on Growth and DNA Damage in Human Cells in Culture

William J Smith, Bethany S Toliver, Janet Moser, Eric W Nealley, Juanita J Guzman and Clark L Gross
Pharmacology Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5400

Biochemical Changes Induced in Human Cells by the Vesicating Agent Sulfur Mustard

William J Smith, Offie E. Clark, Fred M. Cowan, Maura E. DeJoseph, Charisse C. Davenport and Clark L. Gross
Pharmacology Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5400

Imaging Sulfur Mustard Lesions in Basal Cells and Human Epidermal Tissues by Confocal and Multi-Photon Laser Scanning Microscopy

Robert J. Werrlein and Janna S. Madren-Whalley
Pharmacology Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5400

Topical exposure to sulfur mustard (HD) produces separations at the dermal-epidermal junction of human skin and associated blisters following a dose-dependent latent period of 8-24 hours. The mechanisms causing vesication are uncertain. Postexposure images of basal-type keratinocytes indicate that alkylating effects of 400 μM HD (5-min exposures) disrupt molecules that are also disrupted by epidermolysis bullosa-type blistering diseases of the skin. In attached cultures of human epidermal keratinocytes (HEK), there were statistically significant ($p < .01$) decreases of 29.2% and 30.14% in expression of keratins K5 and K14 at 1 hr after exposure, and a nearly complete loss of K14 expression at 2 hrs. Multi-photon imaging showed a corresponding dissolution of keratin filaments, marked by severe loss of cytoskeletal integrity. There were also statistically significant ($p < .01$), non-progressive decreases of 27.3% and 26.3% in expression of α_6 and β_4 integrins at T = 1 hr. Confocal images of cultured cells and multi-photon images of intact epidermis showed depletion of both integrins from the ventral surface of HEK, and dynamic changes in $\alpha_6\beta_4$ receptor structure and cell-surface distribution. Analyses indicate that molecules essential for effective communication and attachment between the basal cell and the basement membrane of skin are early targets of HD-induced vesication.

Suppression of Sulfur Mustard-Increased Il-8 in Human Keratinocyte Cell Cultures by Serine Protease Inhibitors: Implications for Toxicity and Medical Countermeasures

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The toxicity of the chemical warfare blistering agent sulfur mustard (2,2'-dichlorodiethyl sulfide, HD) has been investigated for nearly a century; however, the toxicological mechanisms of HD remain obscure and no antidote exists. The similarity of dermal-epidermal separation caused by sulfur mustard exposure, proteolysis and certain bullous diseases has fostered the hypothesis that HD vesication involves proteolysis and/or inflammation [Reviewed, Cowan & Broomfield, Cell Biol. and Toxicol. (1993) 9, 201]. Although most of the drugs with efficacy for HD toxicity in rodent models act as anti-inflammatory compounds, no in vitro assay is in current use for screening of potential anti-inflammatory HD antidotes. Il-8 is a potent neutrophil chemotactic cytokine that is increased in human epidermal keratinocyte (HEK) cell cultures following exposure to HD and has been proposed as a marker for HD-induced inflammation (Arroyo, et. al., Human & Experimental Toxicol. (1999) 18, 1). Compound screening conducted by the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) established that topical application of four tested serine protease inhibitors could reduce HD toxicity in the mouse ear model. Of theses protease inhibitors ethyl p-guanidino benzoate hydrochloride (in-house identification number, Institute of Chemical Defense, ICD # 1579) was the only compound readily soluble in tissue culture media. N-tosyl-L-lysine chloromethyl ketone (TLCK) is a well-known inhibitor of trypsin-like serine proteases. In the present study, TLCK and ICD # 1579 [1000 to 31.25 μM] were added to HEK cell cultures 1 hr after HD exposure (200 μM) and dose-dependently suppressed HD-increased IL-8. The suppression of HD-increased IL-8 by drug candidate compounds such as protease inhibitors may provide a mechanistic marker that helps predict future medical countermeasures for HD toxicity.

Measurement of Protease Release by a Fluorogenic Casein Assay in Human Cells Exposed in vitro to Sulfur Mustard

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Sulfur mustard (HD) exposure causes severe ocular, respiratory, and blistering injuries to skin (1). The mechanism(s) of injury has eluded identification, although a protease(s) may be involved in the dermal-epidermal separation seen in blister formation. Recent research using *in vitro* systems has demonstrated that protease release occurs in these models (2-4) as a function of HD exposure. A number of protease inhibitors have been proposed as candidate anti-vesicant medical countermeasures and need to be screened in a rapid and cost-effective *in vitro* assay to determine efficacy before being transitioned to an *in vivo* model for further testing. New fluorogenic casein substrates that are sensitive to a number of proteases have been developed by Molecular Probes (5) and may be helpful in developing a rapid assay for protease analysis. This report describes the use of these substrates in assays for screening compounds to develop medical countermeasures against this vesicant agent. The data suggest that these substrates are not appropriate to the development of a rapid protease assay.

Human Keratinocyte Inflammatory Transcriptional Gene Activity Following Sulfur Mustard

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Sulfur mustard (SM) exposure on human skin produces delayed skin vesication at the dermal-epidermal basal adherence contact of the keratinocyte stem cell. The primary changes occurring within the basal keratinocyte layer producing the loss of its attachment are not known. However, a strong, delayed inflammation response occurs in human skin producing large bullous lesions. We were interested in determining changes in inflammation–associated mRNA in cultured

human epidermal keratinocytes (HEK) following SM exposure. In this study, we probed cDNA blots (Human Atlas, Clontech, Palo Alto, CA) at 16 hr and produced subtraction libraries (SL, PCR-Select, Clontech) with cDNA produced from polyA transcripts isolated at 8 and 16 hr after exposure to SM of cultured normal HEK. Exposure was performed either at 25 μM SM, an estimated subvesicating skin exposure, or to 200 μM, a vesicating exposure of SM. The predominant pro-inflammatory transcripts up-regulated in the 16-hr cDNA blot after 200 μM SM were interleukin-8 and macrophage inflammatory protein-2α. Primary cytokines transcripts up-regulated at 200 μM SM were interleukin-1β (IL-1β), interleukin-1α, and tumor necrosis factor-α. None of these transcripts, except IL-1β at diminished levels from control, was expressed at detectable levels at the 25 μM SM exposure. HEK production of these inflammatory transcripts after a high concentration of SM suggests that HEK have a direct role in the events leading to delayed skin blistering by SM.

Prediction of Acute and Skin Chronic Irritation using In Vitro Reconstituted Human Epidermis

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A Model for Prediction of the Dermal Absorption of Hydrophilic Pollutants from Soil

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Estimates of uptake of pollutant compounds by oral, inhalation and dermal exposure routes are required to determine the human health hazards posed by contaminated soils at Army Installations. Exposure assessment for the dermal pathway involves estimation of the area of skin that will receive soil, the amount of soil that becomes adhered to each cm^2 of skin, the concentration of toxicants in the soil, and the efficiency of the transfer of toxicant from soil to skin (% dermal absorption). Previous efforts to develop models for prediction of dermal absorption have met with limited success. However, these efforts have focused on PCBs, TCDDs, PAHs and other very hydrophobic compounds that are considered the most important pollutants at non-military sites. Prediction of the dermal absorption of soil-borne hydrophobic compounds is difficult because it is dependent on complex direct interactions between soil and skin.

The compounds most commonly associated with contamination at military installations differ from the compounds considered most problematic at civilian sites, in that the military compounds tend to be more polar and more water-soluble. Military significant pollutant compounds include nitroaromatics, nitramines, nitrate esters, chemical warfare agents, military herbicides, insect repellents and polar pesticides. Because of the more hydrophilic nature of such compounds it is probable that the predominant pathway of dermal uptake of these compounds involves transfer from the soil to sweat and dermal uptake from the aqueous phase. Thus, dermal absorption of hydrophilic compounds from soil does not require direct interaction between soil and skin, and modeling these events is greatly simplified. The model described herein assumes transfer by the aqueous route and is currently configured to predict uptake as an hourly rate rather than total uptake per exposure event.

The model is being validated for use in risk assessment by comparison of predicted values for dermal absorption with experimental values determined in studies with intact skin.

Subject Analysis and Intellectual Access to Biomedical Information at the Library of Congress: An Overview

R.B. Worobec
Library of Congress, Washington, DC

In Vitro-In Vivo Extrapolation Using Biologically Based Kinetics/Dynamics Modeling

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A major objective of toxicology is to predict the *in vivo* toxicological consequences of human exposure to pure chemicals, complex mixtures and commercial formulations. Historically, the experimental approach to this goal has been to investigate toxicological processes in whole animal models and extrapolate the results obtained to predict human risk using various extrapolation procedures (high dose - low dose extrapolation, interspecies extrapolation and route-to-route extrapolation) and default assumptions. In recent years *in vitro* methods have received greater attention with respect to their possible application in chemical safety evaluation and risk assessment. One major limitation to the broader application of *in vitro* toxicity testing methods is the lack of scientifically validated techniques for the extrapolation of *in vitro* derived data to the *in vivo* situation. Successful approaches to this issue must be based on a solid foundation in mechanistic toxicology and any proposed *in vitro - in vivo* extrapolation procedure can only be rigorously validated in animal models where appropriate experimental studies can be conducted. Ultimately, the validated extrapolation procedures are to be applied to quantitatively predicting target organ toxicity in human populations based on *in vitro* toxicity testing in human cells.

DARPA's Activity Detection Technologies (ADT) Program: Application to Toxicology

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Jennifer A. Reasor, Strategic Analysis, Inc., Arlington, VA

DARPA's Activity Detection Technologies (ADT) Program seeks to develop and demonstrate systems capable of rapidly detecting and extracting information on the biological activity, mechanisms of action, and physiological consequences of exposure of a broad spectrum of agents including chemical toxins, bacterial agents, viral agents, or other agents of interest. Such systems have the potential to rapidly provide important toxicological data about the environment including the presence of a human health risk in the environment, the type or specific identity of an agent present in the environment, as well as the consequences of exposure to such an environment. One of the key advantages to activity based detection systems is their ability to provide information on the presence and toxicity of a broad spectrum of agents including **unknown, engineered, or emerging threats.** Successful development of activity based detection systems could have significant impact in areas such as environmental monitoring, disease surveillance, medical diagnostics, pharmaceutical screening, and clinical trial development (reducing the reliance on animals and humans). One approach to the development of activity based detection systems currently being explored is the fabrication of devices that utilize the unique sensitivity and functional sensory performance of biological cells and tissues. Such systems are designed to measure the perturbation of key molecular processes within cells and tissues after exposure to agents of interest. A number of key research areas are considered to be critical to the successful development of activity detection systems. These include assay development; longevity and stability; chip interface design and engineering; computational design, statistical analysis and data mining tools; sample collection and preparation, and system modeling and integration. Currently, several activity based detection prototype systems have been developed and efforts are under way to test and define the performance capabilities of the prototype systems in both the laboratory and the field. This poster will present an overview of the ADT program, the types of technologies/systems being designed and developed, the six key areas of research that the program focuses on, and the possible impacts of success.

Use of Quantitative Cytotoxicity Data as a Decision-Making Tool for Determining When Chronic Systemic Toxicity Testing is Needed for Medical Device Materials

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Long-term systemic toxicity testing is recommended for some implanted and externally communicating medical devices under the ISO 10993-1 standard. However, such tests are not only costly, but can also use a large number of animals, relative to other biocompatibility tests. Therefore, employing short-term screening techniques would be useful in determining when long-term, *in vivo* toxicity testing is required. CDRH is exploring a two-step process involving a "threshold of toxicological concern" and cytotoxicity data to improve this decision-making process. Although cytotoxicity data are currently used in a qualitative fashion in the biological evaluation of medical devices, recent analyses have suggested that cytotoxicity data can be used in a more quantitative manner to determine when *in vivo* testing is required. Linear regression of quantitative (IC50) data from a number of cytotoxicity studies and LOAEL values from long-term toxicity studies suggested that IC50 values on the order of 35 µg/ml correspond to the proposed threshold of toxicological concern of 0.1 mg/day. Although there are many details to be worked out before such a screening approach can be used in a meaningful way (e.g., cell type, duration of test, endpoint), it appears that quantitative cytotoxicity data could be useful in helping to determine when long-term systemic toxicity testing would be appropriate for medical devices.

Visualize Human Damage & Duration: Toxicology Using Archival Data *

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Sometimes estimates of human hazard levels (and related consequences) are needed when experimental methods can't be used to collect data. Experimental methods might require unavailable human subjects, inappropriate use of animals, or resources and/or time spans that are unreasonable. The best alternative might be to collect diverse results from archived data on accidental human exposures or studies that could not be conducted, now. Useful data might come from intentional but minimal exposures, well established biological parameters, analogous existing animal experimentation, or any other relevant source.

This poster is intended to encourage consideration of existing data as an alternative to experimental use of animals – or man. The approach is illustrated with four examples. One involves estimation of lethal human hazards of hydrogen cyanide inhalation. Another shows how human data on eye effects of mustard gas exposures were compiled for prediction of effects from unknown dosages. The third set shows how degree and duration of incapacitation were projected from viral exposure data. The last example reveals consequences of "Montezuma's Revenge" for a group of persons attending a medical convention – and taking notes.

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Session 2: Neurotoxicity , Mutagenicity and Carcinogenicity

Submillimeter-Wave Frequency Studies of the Vibrational Modes of Deoxyribonucleic Acid: A Metric for Mutagenicity?

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The mutagenicity of a material is one of the more difficult and time-consuming properties to establish. One plausible approach would be to directly monitor the effect of a candidate material on the chromosomes themselves. We have performed a series of

comprehensive experimental and theoretical investigations of the low-frequency internal vibrations associated with Deoxyribonucleic acid (DNA) polymers. The study of the molecular dynamics, achieved via scattering and absorption spectroscopy, is a viable and proven approach that has been applied widely for the general characterization of molecular conformation. Furthermore, there are fundamental physical reasons to expect that measurement and analysis of the spectral data (especially in the very long-wavelength regime) can yield detailed information about complex biological molecules. The submillimeter-wave frequency regime (i.e., ~ 0.01-10 THz) is predicted to be fairly rich with spectral features - DNA phonon modes - that arise out of poorly-localized motions spread over one or more base-pair units. This range of absorption frequencies reflects low-frequency internal helical vibrations involving rigidly bound subgroups that are connected by the weakest bonds, including the weak hydrogen bonds of the DNA base pairs, and/or non-bonded interactions, and vibrations that stretch the bridging hydrogen bonds between the two strands of the biopolymer. These internal motions are extremely sensitive to DNA composition and topology, have an impact on the main processes related to the transfer of genetic information, and eventually can give information regarding the three-dimensional structure and flexibility of the DNA double helix. Furthermore, theoretical studies have predicted DNA phonon frequencies throughout this region, including an optical mode as low as 3 cm^{-1}. Our earlier spectroscopic investigations of DNA films and other biological materials have been performed over a very broad band (300 GHz—100 THz). Free-standing Salmon and Herring DNA film samples with thicknesses between 100 and 400 μm and controlled water content have been prepared and measured, and the results of the measurements demonstrate the existence of multiple dielectric resonances within the biological material at submillimeter-wave frequencies (i.e., ~3-100 cm-1).

Involvement of Actin Cytoskeleton in Acetylcholine Neuroexocytosis and its Inhibition by Botulinum Toxin in Pc12 Cells

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Reorganization of actin filaments has been reported to be a necessary step to regulate Ca^{2+}-dependent neuroexocytosis. Phospholipase A_2 (PLA_2) activation has been implicated in cytoskeletal actin reorganization. We reported that in nerve growth factor (NGF)-differentiated PC12 cells, one of the mechanisms of stimulated acetylcholine (ACh) release inhibition by botulinum toxin A (BoTx) is via interference with our proposed PLA_2-mediated pathway of neuroexocytosis (Ray, P. *et al.*, J. Biol. Chem., 268:11057-11064, 1993; J. Appl. Toxicol., 19:S27-S28, 1999). Recent studies have shown that lysophosphatidic acid (LPA) generated by the action of PLA_2 on membrane phospholipids stimulates Rho family of GTPases to change actin organization in a variety of cells including PC12 cells. Here, we studied the role of actin cytoskeleton in ACh exocytosis and its inhibition by BoTx in PC12 cells. Actin organization was visualized by using the actin specific fluorescent probe, phalloidin. ^{3}H-ACh release was determined as described previously (references above). Both LPA (100 μM) and high K^+ (80 mM) caused actin reorganization and ^{3}H-ACh release. BoTx (100 nM, 4 hr at 37°C) did not have any effect on actin organization, but it blocked the actin reorganization induced by LPA or high K^+, and also the K^+-stimulated ^{3}H-ACh release. Anti-BoTx serum abolished the BoTx inhibition of the LPA-stimulated actin reorganization. These results suggest that actin cytoskeleton is involved in ACh neuroexocytosis and its inhibition by BoTx. We also observed that RhoB was degraded in the toxin treated PC12 cells within 10 minutes after exocytotic stimuli. The 26S proteasome inhibitors prevented this degradation. We conclude that the inhibition of exocytosis by BoTx is due to the degradation of RhoB through the 26S proteasome. Based on these results, we hypothesize that in Ca^{2+}-dependent neuroexocytosis, actin cytoskeleton is involved according to the following scheme: Intracellular free Ca^{2+} increase → PLA_2 activation → generation of LPA → RhoB stimulation → actin disassembly → exocytosis. In BoTx treated cells, actin disassembly and, therefore, exocytosis following stimulation is blocked due to accelerated RhoB degradation via proteasomes.

In CHO Cells Hypothermia Enhances *Bcl*-2 Expression and Protects Against Oxidative Stress Induced Cell Death.

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Oxidative stress is one of the major causes of cellular injury. Various superoxide and nitric oxide radicals are involved in the manifestations of different types of organ toxicity and the resultant syndromes, symptoms or diseases. Hypothermic conditions have been reported to reduce the oxidative stress in various *in vitro* and *in vivo* studies. In the present study, we sought to determine the effect of lowered temperatures on the oxidative stress induced cell death in Chinese hamster ovary (CHO) cells. We also investigated the oxidative stress-induced alterations in the expression of anti-apoptotic protein, *bcl*-2, in CHO cells at lowered temperatures. CHO cells were incubated at 30°C, 32°C, 35°C and 37°C (control temperature) from one to four days. In another set, the cells were incubated with 100 μM hydrogen peroxide (H_2O_2) for 30 minutes before harvesting at different time points. The cells were harvested at one, two, three and four days. Cell survival was significantly higher at 30°C as compared to 37°C over four days of incubation. In cells incubated with H_2O_2, significant cell viability was observed at lower temperatures as compared to the cells incubated at 37°C. The activity of glutathione peroxidase (GSH-Px) also increased significantly at lower temperatures. Lowered temperature also provided a significant increase in the expression of anti-apoptotic protein, *bcl*-2 after four days of incubation. These data suggest that hypothermic conditions lower the risk of oxidative stress induced cellular damage and programmed cell death by increasing the activity of glutathione peroxidase and by the induction in the expression of anti-apoptotic protein, *bcl*-2.

Role of Peroxynitrite and Apoptosis Related Proteins, p53 AND *bcl*-2, in Methamphetamine-Induced Neurotoxicity

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The use of methamphetamine (METH) leads to neurotoxic effects in mammals. These neurotoxic effects appear to be related to the production of free radicals. To assess the role of METH-induced peroxynitrite generation in the dopaminergic cell death pathway, we investigated the production of 3-nitrotyrosine (3-NT) in the mouse striatum. We also sought to determine if the production of 3-NT was related to the expression of the cell death related genes, p53 and *bcl*-2. The levels of 3-NT increased in the striatum of wild type mice treated with multiple doses of METH (4 X 10 mg/kg, 2 hr interval) as compared to the controls. However, no significant production of 3-NT was observed either in the striata of neuronal nitric oxide synthase knockout mice (nNOS-/-) or copper-zinc superoxide dismutase overexpressed transgenic mice (SOD-Tg) treated with similar doses of METH. Moreover, METH treatment up-regulated the expression of p53 and down-regulated the expression of *bcl*-2 in the striatum of wild type mice. No significant alterations were observed in the expression of these proteins in the nNOS -/- and SOD-Tg mice. These data suggest that METH might cause its neurotoxic effects via the production of peroxynitrite and secondary perturbations in the expression of genes known to be involved in the cell death machinery. (Supported by US FDA/NCTR).

Sequence Specific Nucleotide Alterations Induced by MNNG in a Model System of Human Keratinocytes Bearing Integrated SV40 Replication Origins

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Toxicity of Sulfur Mustard to Tetrahymena

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In this report the protozoan Tetrahymena pyriformis was evaluated as an in vitro alternative model to study sulfur mustard (SM) ocular injury. Publications from the cosmetic industry indicated that Tetrahymena could potentially serve as a sensitive in vitro indicator organism. Pending comparable sensitivity to SM, this organism could be used as a platform to rapidly screen potential therapeutic compounds that could modulate toxicity and largely substitute for the Draize test. Normal motion (i.e. motility) of the cells was used as an endpoint for the cytotoxic assay. Based on a series of reproducible experiments it was determined that Tetrahymena had a very high natural tolerance to SM. Concentrations of SM at or above 4mM cytolyzed the cells within a brief period of time while lower concentrations of SM appeared to have limited or no effects. While this result makes Tetrahymena an undesirable in vitro model, it may provide valuable insights into previously unparalleled eukaryotic cellular resistance to SM.

Trophoblast Toxicity Assay

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Many people in the field of mammalian reproduction have concluded that there is a severe lack of information regarding the effects of the majority of drugs and environmental toxins - and the potential detrimental effects of these agents - during pregnancy. The problem has been attributed to the lack of an assay system which can assess the potential side effects of a chemical on the human fetus and placenta. Since the health of the fetus is critically dependent on the quality of placental function during gestation, I have developed an in vitro Trophoblast Toxicity Assay system to evaluate the potential adverse effects that drugs may have on the placenta (and hence fetus) during human gestation. The system utilizes an in vitro trophoblast culture system that I have developed and studied over the last fourteen years (Kliman et al, Endocrinology 118:1567-82, 1986).

Trophoblasts are unique cells derived from the cytotrophoblasts of the outer cell layer of the blastocyst which mediate implantation and placentation (Hertig and Rock, Am J Anat 98:435-494, 1956). My colleagues and I have shown that by studying the differentiation of villous cytotrophoblasts in culture, it is now possible to correlate in vitro trophoblast behavior with in vivo biology of the human trophoblast. Therefore, the basis of the Trophoblast Toxicity Assay system is that drugs and chemicals that alter the differentiated functions of trophoblasts in culture are expected to alter the function of trophoblasts in vivo. Conversely, agents which do not affect a wide array of differentiated trophoblast functions in vitro should not have a deleterious impact on placental function during pregnancy.

In addition to becoming a practical and useful assay to assess drugs and environmental toxins, the further development of the Trophoblast Toxicity Assay may have significant impact on the evaluation of compounds that have exposure potential during pregnancy and could possibly become a standard safety assay for gestational therapeutics.

The Use of Reconstituted Human Corneal Epithelium as Alternative for In Vivo Draize Eye Irritation Testing

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An In Vitro Model System for the Analysis of Toxicant Effects on Neuronal Function

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A new in vitro model system, involving analysis of membrane electrical characteristics of NG108-15 cells is being applied to the examination of toxicant action on neuronal function. In the current study, the effects of the nerve agents soman, sarin and VX and the vesicant sulfur mustard (HD) on this model system were determined. Concentration-dependent effects of these agents on membrane potential, input resistance and the generation of action potentials in response to electrical stimulation were analyzed. HD (10 - 300 μM) was found to produce a rapid (within min) and sustained depolarization of NG108-15 cells. Alterations in action potential waveform or the complete loss of induced action potentials were also evident with 10 - 300 μM HD, whereas significant reductions in input resistance were detected at 30 - 300 μM. These data indicate that HD produces substantial alterations in membrane electrical characteristics at concentrations well below those required for cytotoxic effects in many cells and may thus contribute to the neurological dysfunction produced by HD in vivo. In contrast, the nerve agents soman and sarin (0.01 - 10 μM) and VX (0.03 - 3 μM) were found to produce only minor changes in NG108-15 cell membrane electrical characteristics even at relatively high concentrations. Neither membrane potential nor input resistance was significantly altered ($p>.05$) by these compounds. However, with the highest concentrations of soman or sarin (10 μM) and VX (3 μM) some individual cells were slightly depolarized (by 6-8 mV) or exhibited enhanced membrane input resistance. Slight alterations in action potential waveform were also present in cells depolarized by the nerve agents.

The results obtained with HD confirm the utility of this model system for the analysis of the toxic effects of some neuronally active compounds. However, the finding that nerve agents did not produce marked alterations in electrical characteristics of NG108-15 cells indicates that this system is not useful for the study of all neuronally active compounds. The absence of marked nerve agent-induced alterations in membrane electrical characteristics in this cholinergic cell line may be due to the immature synapses formed by NG108-15 cells and a relatively low level of synaptic activity in the cultures. This model therefore may not be appropriate for the analysis of toxic agents that require efficient connectivity for their action.

Innervating the Corneal Equivalent

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The cornea contains a denser network of nerves than any other organ in the human body. This innervation plays important roles in the maintenance of corneal structure and function. A loss of nerves has been shown to slow corneal wound healing and contribute to dry eye. If nerves could be grown within the artificial cornea (Griffith et al., (1999) Science 286:2169-2172), this model could be a useful tool in studying corneal innervation. Also, an innervated functional artificial cornea would be useful in pain and toxicity testing. Corneal equivalents were constructed using immortalized human corneal cells grown within a collagen-based matrix that acts as a scaffold. Dorsal root ganglia (DRG) isolated from chick embryos were co-cultured with the cornea. It was found that modifications to the corneal matrix with other extracellular molecules, retinoids and growth factors promoted neuronal growth. It was shown that laminin was important for neurite outgrowth into the matrix and retinoic acid enhanced nerve fascicle development. Both nerve growth factor and the presence of stromal keratocytes promoted neurite outgrowth. A high concentration of sub-epithelial nerves was observed. This is similar to the morphology of a human cornea's sub-epithelial nerve plexus. Continued research with this model could lead to an artificial tissue for in vitro toxicology, an alternative to animal testing and could be used to elucidate the role nerves play in corneal wound healing.

Assessing Functional Recovery after Chemical Exposure in Cultured Porcine Corneas: An Ex Vivo Model for Alternative Ocular Irritation Tests

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A Microassay Method Using a Neuroblastoma Cell Line to Examine Neurotoxicity of Organophosphate Mixtures

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We have examined the applicability of using a 96-well plate format to evaluate the toxicity of mixtures of organophosphate (OP) compounds. An assay for acetylcholinesterase (AChE) activity using an immortal neuroblastoma cell line, SH-SY5Y, was performed in the presence of two OPs, paraoxon and dichlorvos. Exposure to either paraoxon (10^{-7} M – 10^{-13} M) or dichlorvos (10^{-5} M – 10^{-8} M) resulted in a dose-dependent decrease in AChE activity by the neuroblastomas. This inhibition was significant at concentrations above 10^{-10} M paraoxon and 5 x 10^{-7} M dichlorvos. To assess the effects of combinations of these OPs, three concentrations of each were selected: 10^{-9} M, 10^{-10} M, and 10^{-11} M paraoxon and 10^{-6} M, 10^{-7} M, and 10^{-8} M dichlorvos. Included were concentrations that significantly inhibited AChE activity, and concentrations that did not. In general, combinations of these OPs inhibited AChE activity in an additive manner. In each mixture that was significantly different from the control, at least one of the individual components also significantly inhibited AChE activity. Results with the individual OPs are in agreement with similar studies from other laboratories using the SH-SY5Y cell line. The results from the single compounds, together with those of the combined OPs, suggest that this assay can be used effectively as a rapid screening method to assess the effects of mixtures on AChE activity.

***In vitro* Neuromolecular Analysis of Trimethylolpropane Phosphate, a Model Toxicant**

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Cell-based biosensors utilizing mammalian cells offer the promise of generic, physiologically-relevant sensitivity to a wide range of environmental threats. Our laboratories are exploring the utility of cultured neurons for the detection and classification of neurotoxicants via state-of-the art methods in electrophysiology and genomics. Trimethylolpropane phosphate (TMPP), a pyrolysis product from synthetic ester turbine engine lubricant and certain flame retardant foams, constitutes a potential occupational environmental threat. Past work has demonstrated that exposure to TMPP induces epileptiform activity in animal neurobehavioral models. Using cortical neuron-glia co-cultures grown on microelectrode array substrates, we observed that TMPP reproducibly increases mean spike rate and alters burst profile and patterns in a manner consistent with blockers of inhibitory synaptic transmission capable of triggering seizure activity. To validate and extend these findings, molecular analysis achieved via the whole-cell patch clamp method revealed that TMPP depresses γ-aminobutyric type A receptor function. Efforts to develop a portable, prototype cell-based biosensor capable of monitoring neural network extracellular potentials will be described. These data complete a broad spectrum analysis of a model toxicant spanning whole animal to molecular levels and suggest that neurotoxicant detection and classification using cell-based biosensor systems may soon be possible. In addition, preliminary gene expression analysis of TMPP exposed brain tissue will be presented. Future work involving the extension of genomics technology to cultured in vitro systems and challenges for routine use of cell-based biosensor systems will be identified.

Use of a Novel Clonal Murine Bone Marrow Stromal Cell Line (D2XRIIGFP24) as a Fluorescent Cytosensor for Assessment of Genotoxic Stress

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D2XRIIGFP24 cell line was cloned to assess genotoxic activity of physical, chemical,

and biological factors (such as ionizing/non-ionizing radiation, oxidants, polycyclic aromatic hydrocarbons, depleted uranium, antibiotics, etc.) using on-line digital fluorescence imaging, and/or flow cytometry analysis. The biological plausibility of using this cell line is based on a p53(15x)-d2EGFP reporter vector employed for the transfection of the precursor D2XRII cells. p53(15x)-d2EGFP vector contains the green fluorescent protein (EGFP) reporter and upstream Tp53 enhancer elements driving expression of EGFP. Since activation and/or expression of Tp53 are strongly associated with DNA damage, the genotoxic stress to D2XRIIGFP24 cells is followed by increase in the EGFP fluorescence. Thus, the EGFP response was observed after exposure of D2XRIIGFP24 cells to hydroperoxide, UVB light, and ionizing radiation. This effect was dose-dependent, and with temporal sequences that were dependent on the nature of the agents. The Tp53-dependent increase in EGFP fluorescence was corroborated by Western blot analysis of Tp53, and the comet assay for damaged DNA in D2XRIIGFP24 cells.

Cultured Sympathetic Neurons as a Model System for Investigating the Developmental Neurotoxicity of Organophosphate Pesticides

P.J. Lein, R.A. Schuh, R. Bucelli*, and D.A. Jett, Environmental Health Sciences, Johns Hopkins University, Baltimore, MD and *Biology, Canisius College, Buffalo, NY

There is increasing evidence that cognitive and behavioral problems may be linked to perinatal exposure to organophosphate pesticides (OPs). However, delineating the mechanism(s) by which OPs induce these changes in neuronal function is difficult since there is limited data available regarding the stage(s) of neuronal development targeted by OPs. It has been proposed that developmental neurotoxicants such as the OPs may cause functional deficits by interfering with morphogenic events critical to establishing neuronal connectivity, specifically axonal and dendritic outgrowth and synaptogenesis. The effect of toxicants on these events is difficult to study in the intact animal; thus we are evaluating the use of cell culture systems to identify and characterize mechanisms of actions of toxicants that perturb axonal and dendritic morphogenesis. Cultured sympathetic neurons were chosen as a model system because there has been extensive characterization of the endogenous factors that regulate morphogenesis of these neurons both in situ and in vitro. Moreover, dissociation of sympathetic neurons from the superior cervical ganglia of perinatal rat pups yields a homogenous population of neurons that can be maintained for up to 2 months in culture in the absence of glia and serum. These cells can be grown under conditions in which they extend only a single axon and no dendrites, or a single axon and a dendritic arbor comparable in size to that extended by in vivo counterparts, thus it is possible to screen for agents that induce de novo dendritic growth or that inhibit dendritic growth in response to physiological inducers of dendritic growth. We have preliminary data indicating that a 24 hr exposure to chlorpyrifos (CPF, 0.01 to 10 μM) or its oxon metabolite, CPFO (0.01 to 10 nM) results in a significant inhibition of axonal outgrowth in sympathetic neurons as measured with respect to the percentage of neurons with axons, the number of axons per neuron, and total length of the axonal plexus. Measurements of cell viability and AChE catalytic activity suggest that inhibitory effects on axon outgrowth occur independently of effects on either of these parameters. In contrast, a 72 hr exposure to the same concentration range of these OP pesticides causes a significant enhancement of dendritic growth in these neurons, even in the absence of physiological inducers of dendrite formation. These data suggest that this model system is useful for identifying effects of toxicants on neuronal morphogenesis. Furthermore, these data indicate that toxicants may exert differential effects on axonal and dendritic growth.

Changes in Gene Expression After Exposure to Organophosphorus (OP) Agents

Jennifer W. Sekowski[1], Kevin P. O'Connell[1], Akbar S. Khan[1], Maryanne Vahey[2], Martin Nau[2], Maha Kahlil[3], Mohyee E. Eldefrawi[3], and James J. Valdes[1]
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The acute effects of chemical weapons have been widely studied since World War I and are fairly well understood. Since the Gulf War, however, there has been increased concern for exposure of personnel to sub-acute and low-levels of chemical warfare agents. Additionally, questions have been raised as to the effects of exposure to toxic industrial chemicals (TICs) and materials (TIMs) and other environmental contaminants during deployments to countries where agricultural chemicals (pesticides, herbicides) are virtually unregulated and chemical manufacturing and disposal methods are outdated. Although sub-acute exposures may not cause obvious pathology at the time of exposure, they may cause genetic alterations or changes in gene expression that may predispose personnel to disease later in life.

In this pilot study, Sprague-Dawley rats were injected with sub-LD50 doses of the organophosphorus (OP) warfare agent, Sarin (GB), the OP insecticide, chlorpyrifos (CPF), or a Hepes-buffered saline control. After extracting the RNA from the livers and brains of the animals at 1,4, or 24 hours post-exposure, the level of gene expression was assessed using DNA microarray analysis. While our studies of GB are ongoing, it appears that exposure to CPF results in several significantly altered patterns of gene expression. The patterns of altered gene expression and the specific classes of affected genes will be discussed.

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Wednesday, November 29

Welcome

Mr. Mike Parker
Deputy to the Commander
US Army Soldier and Biological Chemical Command

Keynote Address
Moving Environmental Health Sciences into the Twenty-First Century

Anne P. Sassaman, Ph.D.
Director, Division of Extramural Research and Training
National Institute of Environmental Health Sciences

The mission of the National Institute of Environmental Health Sciences (NIEHS) is to reduce the burden of environmentally-associated disease and dysfunction by defining 1) how environmental exposures affect human health, 2) how individuals differ in their susceptibility to these exposures, and 3) how these susceptibilities change over time. Now that the human genome has been sequenced and prospects are bright for the application of this knowledge to the understanding of human disease, it is becoming more and more apparent that genetics alone are not sufficient to explain most diseases. We must understand the complex interaction of one's genes with one's environment and how individuals vary in their response to the environment. The NIEHS defines "environment" very broadly and is prepared to develop new initiatives to explore the influence of diet and nutrition, dietary supplements, and social environment, for example, as they relate to health or as they modify exposure to agents more traditionally considered in toxicology. The Institute has also identified a number of areas in which strategic investments must be made in order to improve environmental decision-making, which depends upon good science as its base. An example of this is the new National Center for Toxicogenomics, a major investment in the use of mRNA expression and proteomic approaches to advance our understanding of the impact of environmental exposures. These technologies will also provide a new approach to testing and characterizing chemical and physical agents and hold the promise of revolutionizing traditional toxicology. The Institute's strategic investments and new directions will be discussed in the context of meeting the challenges of the new millennium for environmental health in general, and toxicology in particular.

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Progress in the Validation and Regulatory Acceptance of Alternatives

Session Chairs: Neil Wilcox, D.V.M., Gillette Company and
William Stokes, D.V.M., NIEHS

Introduction and Overview of the Session

Neil Wilcox, D.V.M.
Gillette Company

Update on the ICCVAM

William Stokes, D.V.M.
National Institute of Environmental Health Sciences

Validation and Regulatory Acceptance of Alternative Test Methods: Current Situation in the European Union

Julia Fentem, Ph.D.† and Michael Balls, M.A. DPhil FIBiol‡

†SEAC Toxicology Unit, Unilever Research Colworth, Sharnbrook, Bedfordshire MK44 1LQ, UK; ‡ECVAM, Institute for Health & Consumer Protection, European Commission - Joint Research Centre, I-21020 Ispra (VA), Italy

On 4th February 2000, the European Union (EU) Member States approved the first replacement alternative (*in vitro*) methods to be mandated for use for regulatory toxicity testing. *In vitro* tests for *skin corrosion* (the rat skin transcutaneous electrical resistance [TER] method and tests employing human skin models; Fentem *et al.*, 1998; Liebsch *et al.*, 2000) and *phototoxicity* (the 3T3 neutral red uptake [NRU] phototoxicity test; Spielmann *et al.*, 1998a; 1998b) had both been shown unequivocally to be reliable and relevant in extensive prevalidation and formal validation studies conducted under the auspices of the European Centre for the Validation of Alternative Methods (ECVAM). Following critical, independent, peer review, the validities of these replacement alternatives were endorsed by ECVAM's Scientific Advisory Committee (ESAC; ECVAM, 1998a; 1998b), the Scientific Committee on Cosmetic Products and Non-Food Products intended for consumers (SCCNFP), and the relevant services of the European Commission (EC; DG Environment and DG Enterprise) during 1998. Draft test guidelines on skin corrosion and phototoxicity were then submitted to the OECD in the latter part of 1998. After more than a year of inactivity on these draft guidelines at OECD level, the EC decided as an interim step to discuss the new tests with the EU National Coordinators for test methods, with a view to their incorporation into Annex V of the Dangerous Substances Directive (DSD; *67/548/EEC*). This was achieved via *Commission Directive 2000/33/EC* of 25 April 2000, the 27th adaptation to technical progress of *Council Directive 67/548/EEC* (EC, 2000), which incorporates test methods "B.40 Skin Corrosion" and "B.41 Phototoxicity - *In Vitro* 3T3 NRU Phototoxicity Test" into Annex V of the DSD, making their use mandatory within the EU.

As a follow-up to the successful ECVAM validation study on *in vitro* tests for skin corrosion, a prevalidation study on *in vitro* tests for acute skin irritation has been undertaken during 1999 and 2000 (Fentem *et al.*, 1999; 2000). The outcome of this study will be outlined briefly in the presentation, as well as highlighting other key studies, initiatives and on-going activities in the EU (principally coordinated by ECVAM) relevant to the validation and regulatory acceptance of *in vitro* tests for eye irritation, skin penetration, embryotoxicity and haematotoxicity.

In addition to reviewing studies and activities emanating from the EU, the ESAC has also reviewed the submission on the mouse local lymph node assay (LLNA) made to ICCVAM, endorsing the validity of the LLNA as a reduction and refinement alternative for skin sensitisation testing (ECVAM, 2000). In December, the ESAC will review the submission on Corrositex™ made to ICCVAM, and the findings of the ICCVAM Peer Review Panel. Thus, the EU now has in place a process for discussing and endorsing the validities of alternative methods evaluated, without direct ECVAM involvement, anywhere in the world. Once such processes for mutual recognition of validated methods have also been agreed within other key countries/regions, we should begin to see more timely and efficient progress in implementing validated alternative methods into regulatory testing requirements at an international *cf.* national/ regional level.

EC (2000) *Official Journal of the European Communities* **L136**, 90-107
ECVAM (1998a) *ATLA* **26**, 7-8
ECVAM (1998b) *ATLA* **26**, 275-280
ECVAM (2000) *ATLA* **28**, 366-367
Fentem *et al.* (1998) *Toxicology in Vitro* **12**, 483-524
Fentem *et al.* (1999) *Alternatives to Animal Testing II. Proceedings of the Second International Scientific Conference Organised by the European Cosmetic Industry, Brussels, Belgium, 1999*, pp. 228-231
Fentem *et al.* (2000) *Toxicology in Vitro*, in press
Liebsch *et al.* (2000) *ATLA* **28**, 371-401
Spielmann *et al.* (1998a) *Toxicology in Vitro* **12**, 305-327
Spielmann *et al.* (1998b) *ATLA* **26**, 679-708

Integrated In Vitro Approaches for Assessing Acute Toxicity

Anna Forsby, Ph.D.
Department of Neurochemistry and Neurotoxicology, Stockholm University, SE-106 91 Stockholm, Sweden

Cellular toxicity data determined *in vitro*, were integrated with computer models based on *in vitro*-derived biokinetic data. A set of eight well characterized test compounds, which specifically affected the central or peripheral nervous system, was selected. The compounds were tested in an *in vitro* test battery, which were composed of assays measuring general and neurospecific endpoints. Basal cytotoxicity, protein synthesis rate (PSR) and basal intracellular free Ca^{2+} concentration (basal Ca^{2+}) were determined as general endpoints. Neurospecific endpoints were; neurite degeneration (ND), voltage operated Ca^{2+} channel (VOCC) function and acetylcholine receptor (mAChR) signal transduction measured by high $[K^+]$- evoked and carbachol-activated Ca^{2+} flux, respectively. Differentiated human neuroblastoma (SH-SY5Y) cells were used for the studies. The cellular neurotoxic activities for six of the eight test compounds were determined after 72 hrs of exposure by measuring effects on the endpoints. Toluene and n-hexane could not be tested in the battery due to technical obstacles such as volatility and reaction with the plastic used for the cell cultures. By using values for the concentrations that induced 20 % effect (EC20) in the most sensitive endpoint, it was possible to establish critical neurotoxic concentrations (CNCs). The CNCs were hypothesized to mimic the lowest toxic target tissue levels. The CNC for each compound was integrated in a computer-based biokinetic model for rat and lowest effect doses (LOEDs) after acute and/or subchronic exposure were estimated. Due to bioactivation of parathion to paraoxon, the estimated LOED for parathion was determined by integrating CNCs for paraoxon in the model. For acrylamide and lindane, the study was extended by the integration of additional EC20- or EC50-values from other endpoints than those generating CNCs, in order to investigate the possibility to estimate doses for acute systemic toxicity and subchronic convulsions (lindane). The estimated doses were validated by comparison with experimentally derived *in vivo* doses, found in literature (table 1).

Table 1. Doses estimated from neurotoxicity *in vitro* data as compared to experimentally derived neurotoxic doses from literature for six test compounds

Compound	*In vitro* endpoint	*In vivo* endpoint; exposure[a]	Estimated dose[b]	Experimental dose[b]
Acrylamide	20% cytotoxicity	gait; a.	73	75
Acrylamide	CNC; ND	startle response; sc. 10 d.	23	15
Acrylamide	CNC; ND	startle response; sc. 30 d.	9.3	10
Acrylamide	CNC; ND	startle response; sc. 90 d.	3.6	3.3
Caffeine	CNC; inh. VOCC	anti-nociception; a.	3.9	12
Diazepam	CNC; inh. VOCC	time to emerge; a.	115	0.01
Lindane	CNC; inh. VOCC	learning; sc.	1.4	2.5
Lindane	CNC; incr. basal Ca^{2+}	convulsions; sc.	16	12
Lindane	50% cytotoxicity	lowest LD50; a.	210	90
Lindane	50% cytotoxicity	highest LD50; a.	210	270
Parathion/ paraoxon	CNC; ND (paraoxon)	tail-pinch response (parathion); a.	4.2	2.0
Phenytoin	CNC; inh. of PSR	operant learning; a.	74	10

a) investigated after acute (a.) or subchronic (sc.) exposure
b) LOED (mg/kg or mg/kg/day) or LD50 (mg/kg)

A good correlation between estimated and experimental toxic doses were obtained for all compounds but diazepam, for which the correlation between estimated and experimental

LOED differed about 10,000 times. The reason was that the cellular mechanism ($GABA_A$ receptor-activation) most probably underlying the experimental *in vivo* effect (time to emerge) after acute exposure, was not included in our neurotoxicity test battery. When literature data on diazepam-activated $GABA_A$ receptor-function was integrated in the biokinetic model, a good estimation of LOED (0.09 mg/kg) as compared to experimental LOED (0.01 mg/kg), was obtained.

Validation and Regulatory Status of Alternative Methods to Replace the Conventional LD50 Test

Kathy Stitzel, Ph.D.
Procter & Gamble Company

The OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation

Errol Zeiger, Ph.D.
National Institute of Environmental Health Sciences, Research Triangle Park, NC

This month, the Environmental Directorate of the Organisation for Economic Co-operation and Development (OECD) will be releasing its new Guidance Document, (No. 19) Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Humane endpoints are defined as the earliest indicators in an animal of severe pain, distress, suffering, or impending death. This document defines these terms and addresses the concept of refinement of animal testing through the use of humane endpoints. Procedures that can be put in place to minimize animal pain, distress, and suffering during regulatory toxicity testing are described. The Guidance Document emphasizes the need for careful design and monitoring of experiments, and expert judgement in the application of humane endpoints. The goal of the experimenter should be to use humane endpoints to minimize pain, distress, or suffering to the extent possible without compromising the scientific objectives of the experiment. The document provides guidance on the humane conduct of specific types of toxicity tests, including acute and long-term tests. It also contains an extensive listing and description of clinical signs that should be considered as indicators of pain or distress or impending death.

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Development of Predictive Methods Based on Mechanisms of Eye Irritation at the Ocular Surface: Meeting Industry/Regulatory Needs

Session Chairs: Sherry Ward, Ph.D., Gillette Company and Wiley Chambers, M.D., FDA

Introduction & Symposium Overview and Goals

Sherry Ward, Ph.D.
Gillette Company

Industry Perspective - Meeting Industry Needs for Draize Alternatives

Leon Bruner, D.V.M.
Gillette Company

Pathophysiology and Clinical Features of Thermal and Chemical Eye Injuries

Martin Reim, Professor Dr. med.
Technical University, Aachen
Aachen, Germany

Evaluation and Refinement of the Bovine Cornea Opacity and Permeability (BCOP) Assay

John L. Ubels, Ph.D.
Department of Biology, Calvin College, Grand Rapids, MI

Phillip L. Casterton
Amway Corporation, Ada, MI

The BCOP assay has been proposed as an *in vitro* method for predicting ocular irritancy or toxicity of chemical substances. In this assay, bovine corneas are mounted between two saline-filled chambers. After exposure to a potential irritant, absorbance of light and permeability to fluorescein are measured to assess damage to the cornea. Our goal is to refine and validate this assay based on principles of corneal physiology. Corneal opacity, or a loss of transparency, can be the result of an increase in corneal hydration or direct damage to corneal tissue. We have studied the role of changes in corneal hydration in increased opacity measurements. Increases in corneal hydration have a limited effect on cornea opacity as measured in the BCOP assay. Even a maximally hydrated cornea has an opacity that would yield an irritancy ranking in the moderate range. In contrast, severe irritants may cause maximal opacity of the cornea with relatively small increases in hydration. This suggests that increases in opacity in excess of that caused by increased hydration are indicative of tissue damage. It is suggested that measurement of corneal hydration be added to the BCOP assay to permit assessment of mechanisms of tissue damage.

Our studies of the BCOP assay have also included the use of reduced exposure times to chemicals. The standard exposure time of 10 minutes is designed to yield results that correlate with Draize test data. This may lead to an overestimation of ocular irritancy. Our studies of the use of exposure times of 30 seconds or 1 minute show reduced opacity and hydration levels in response to most test substances. Use of reduced exposure times in the BCOP assay may be more predictive of human response to ocular irritants.

Finally, we have studied effects of chemical irritants on the corneal endothelium, a cell layer that is essential to normal corneal function and transparency. These cells are non-proliferative and therefore knowledge of their condition is essential to prediction of recovery from corneal injury. Following removal of the cornea from the holder, the morphology and viability of the corneal endothelium can easily be assessed by staining with Alizarin Red S and trypan blue. Our studies show that severe irritants can penetrate the cornea and damage the endothelium; however, the corneal holder used in the BCOP assay itself causes extensive damage to the endothelium. This holder clamps the non-spherical bovine cornea into a circular opening between the chambers causing extensive wrinkling of the tissue and loss of 20% of endothelial cells. Using computer-aided design we have developed a corneal holder appropriate to the dimensions and curvature of the bovine cornea. The internal structure of the holder is designed so that no contact is made with the corneal epithelium or endothelium. Normal corneal curvature is maintained and no wrinkling occurs during mounting or incubation of the corneas for 3 hours. Following incubation the endothelium is undamaged. Development of this holder represents an important advance in BCOP assay technology. The design program for the holder will be made available to research and industrial laboratories.

The Use of Corneal Organ Culture as an Ex Vivo Model for Ocular Toxicity Test of Commercial Hair Care Products

Fu-Shin X. Yu, Ph.D. and Ke-Ping Xu, M.D.
Schepens Eye Research Institute
Harvard University

We previously reported the use of a simple air-lifted corneal organ culture as an ex vivo model, measurements of corneal epithelial permeability and DNA-binding activity of stress-response transcription factors following surfactant exposure as parameters for an ocular irritancy test. Using this approach, we report here the evaluation of potential toxicity of three hair care products, termed GA, GB and GC, in a double-blinded manner. Both GA and GB induced tight junction disruption and increased permeability of corneal epithelium which was

assessed using surface biotinylation in a concentration and time-dependent manner. A decrease in DNA-binding activity of transcription factors, measured using electrophoretic mobility shift assay, was observed when cultured corneas were treated with GA and GB at concentrations likely to cause ocular irritation. However, GC caused neither tight junction disruption nor changes of DNA-binding activity of AP-1 and NF-□B, suggesting minimal irritancy of the product. Thus, our ex vivo testing correctly distinguished one mildly irritating product (GC, Draize score 14.2) from those causing moderate irritation (GA and GB, Draize scores 37.8 and 57.4, respectively). These results indicate that corneal organ culture, in combination with measurements of toxicant-induced stress responses at organ, cellular and protein levels, has the potential to be used as a mechanistically based alternative to in vivo animal testing of consumer products.

Extent of Corneal Injury as the Mechanistic Basis for the Development of Alternative Ocular Irritation Tests

James V. Jester, Ph.D.[1] and James K. Maurer, D.V.M., Ph.D.[2]
[1]University of Texas Southwestern Medical Center at Dallas, Dallas, Texas and [2]The Procter & Gamble Company, Cincinnati, Ohio.

We have characterized ocular irritation to a wide range of irritants differing in type (surfactants, acid, alkali, bleaches, alcohol, aldehyde, acetone) and severity (slight to severe) using the low volume rabbit eye test. Light microscopy was used to detail the pathological changes in the cornea, conjunctiva and the iris/ciliary body over time. *In vivo* confocal microscopy (CM) was used to quantify 4-dimensionally (x, y, z and t) initial corneal injury and later responses in the same eye, and laser scanning CM to quantify initial corneal cell death. These studies revealed that regardless of the processes leading to injury, slight irritants injure the corneal epithelium, mild/moderate irritants injure the corneal epithelium and the anterior stroma, and severe irritants injure the epithelium, deep stroma, and at times the corneal endothelium. Moreover, extent of initial corneal injury was shown to predict subsequent responses and final outcome. Our findings indicate that a broad, mechanistically based alternative ocular irritation test to eliminate the use of live animals should be comprised of either an *ex vivo* or *in vitro*, 3-dimensional corneal equivalent system, for which extent of injury could be measured by either microscopic or biochemical techniques.

To test the validity of this approach we have used an *ex vivo*, rabbit cornea culture model to measure extent of corneal injury following exposure to a variety of ocular irritants. Corneal buttons (8.5 mm) obtained from abattoir rabbit eyes were placed on agarose culture dishes at an air-liquid interface. After overnight culture, buttons were exposed to different ocular irritants of known severity by applying the materials to a sterile, 3 mm diameter filter paper disk, and then placing the disk on the cornea for 10 seconds. Buttons were subsequently washed and cultured for 3 hrs, 24 hrs and 48 hrs after exposure. Extent of injury and cell death were measured by laser scanning CM. Importantly, the extent of injury observed in the *ex vivo*, organ culture model significantly correlated with the extent of initial injury measured previously in live animals using similar techniques. These finding support further our hypothesis that extent of initial corneal injury can be used as a basis for the development of broad, mechanistically based alternative eye irritation tests.

Lastly, our studies have identified 3 critical considerations. 1.) Replacement tests should be able to differentiate injury which is diffuse (e.g., surfactants) from that which is focally extensive (e.g., alkali); 2.) Maximal initial injury may occur at different times depending on the irritant (e.g., surfactants vs. formaldehyde) requiring assessment of multiple time points to accurately predict ocular irritation potential; and 3.) While most irritants show progressive injury extending through the epithelium into the stroma, some irritants (e.g., bleaches) cause a disproportionate amount of stromal injury with limited epithelial injury, emphasizing the importance of including both epithelial and stromal components in an alternative model.

Area and Depth of Injury in a Rabbit Ex Vivo Corneal Model

Rosemarie Osborne, Ph.D.
Procter & Gamble Company

Engineered Tissue Equivalents for Ocular Irritancy Screening

May Griffith, Ph.D.
Ottawa General Hospital

The EpiOcular Prediction Model: A Reproducible In Vitro Means of Assessing Ocular Irritancy

Patrick J. Hayden, Ph.D.,
J. Kubilus and M. Klausner
MatTek Laboratories, Ashland, MA

EpiOcular™ (OCL-200) is an organotypic tissue model of the human corneal epithelium (HCE) cultured from normal human keratinocytes using serum free medium. H&E stained histological cross-sections show that the structure of EpiOcular closely parallels that of the HCE. Previously, a prediction equation for eye irritation, Draize score (MMAS) = -4.74 + 101.7/ √ET-50, was developed by correlating the *in vitro* ET-50 with Draize rabbit eye scores for 19 water-soluble chemicals from the ECETOC database and 41 cosmetic or personal care products/ingredients (ET-50 refers to the time of exposure which reduces the tissue viability to 50%, as determined by the MTT assay, in minutes). The current study reports *in vitro* results for an additional 24 consumer products, including shampoos, hand soaps, laundry detergents, dishwashing liquids, and skin lotions. A plot of the *in vivo* and calculated *in vitro* Draize scores, when correlated to the line *In Vivo Draize (MMAS) = In Vitro Calculated Draize*, gave the correlation coefficient, r = 0.85; when a single outlier was excluded r = 0.93. The useful range of the EpiOcular test was evaluated by testing surfactants at concentrations at which the Draize test is insensitive (MMAS scores < 2.0). EpiOcular was able to distinguish between surfactants at concentrations 3-10 fold below this point. Thus, the EpiOcular tissue model appears to be a sensitive, accurate *in vitro* means of predicting *in vivo* ocular irritancy for a range of consumer products and raw materials.

Cell Biology of Immortalized Human Corneal Epithelial Cells in 3-Dimensional Cultures for In Vitro Toxicology Assays

Roger Beuerman, Ph.D.
Louisiana State University Health Sciences Center,
Doan Nguyen, Bart De Wever

Performance of the HCE-T TEP Human Corneal Epithelial Transepithelial Fluorescein Permeability Assay

Sherry Ward, Ph.D. (Gillette Company)

Regulatory Perspective - Meeting Regulatory Agency Needs for Draize Alternatives

Wiley Chambers, M.D.
US Food and Drug Administration

Science has caught up with the Draize Rabbit Eye Test. From a regulatory perspective, it is not "the gold standard" and is no more or less useful than any other un-validated in vitro or in vivo model. Correlation of new testing methodology with the Draize Rabbit Eye Test has limited utility because the Draize Test itself is not predictive of human injury. New testing methodologies should be compared to human testing. Human testing should be prospectively planned for products which are expected to lead to human exposures, but not expected to cause human injury. Accidental exposure information for products expected to cause human harm should be collected using a pre-planned data collection system.

Dinner Speaker:

James MacGregor, Ph.D., D.A.B.T.
FDA Center for Drug Evaluation and Research

The Biotechnology Revolution and the Evolution of Regulatory Toxicology

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Dermal

Session Chairs: William J. Smith, Ph.D.,US Army MRICD and
Larry Rheins, Ph.D., DermTech International

Validating *in vitro* Dermal Absorption Studies, an Introductory Case Study

Robert P. Zendzian, Ph.D. and
Michael Dellarco, Dr. PH
United States Environmental Protection Agency,
Washington D.C. 20460

In vitro dermal absorption studies have been used for 40-50 years in attempts to understand the process of dermal penetration and to quantify the passage of particular chemicals across mammalian skin. However questions remain as to the validity of *in vitro* methodology in relation to the *in vivo* process. The Office of Pesticide Programs (OPP) of the U S Environmental Protection Agency has received a number of matched *in vivo* and *in vitro* dermal absorption studies of pesticides in the rat as part of the pesticide registration process. The data set on Acetochlor will be presented as an example of the experimental design and protocol that we consider necessary to allow one to evaluate the validity of an *in vitro* procedure. Acetochlor was tested at four common doses and six common durations of exposure *in vivo* and *in vitro*. This allows comparison of both dose and time related variation of dermal penetration. Four rats were used per dose duration *in vivo* and 4 to 7 samples of the isolated rat epidermal membrane preparation were used per dose duration *in vitro*. The original pesticide Registrant submitted reports of these two studies presented in detail: the experimental design, dose preparation, animal/skin sample preparation, dose administration, sample collection and sample analysis. All raw data generated in the studies were reported in tabular form. The data clearly show that this particular *in vitro* procedure in the rat greatly over estimated dermal absorption in the majority of doses and exposure durations with the greatest error in the lower doses. The over estimate ranged from 2 to 7 fold.

Utility of Gene Array Technology in Skin Biology

Brian Jones, Ph.D.
Avon Products, Inc., Suffern, NY

The rapid advancement of molecular biology and wealth of DNA data generated by th Human Genome Project has provided a means to utilize genomic information, by way of gene array technologies, to elucidate basic areas of biology. Gene array technology can provide a powerful method to gain insight into gene function and gene pathways by determining gene expression profiles of normal and disease states. These profiles have the potential to be established for most, if not all, cellular and organ systems within the body. The method has already been used to provide insight into the pathophysiology of cancer, leading to possible methods of early diagnosis, therapy, and even prevention. Gene array technology is a rational, "next-step" approach into making significant gains in the basic knowledge of skin biology. Description of the basic types of array methodology (membranes/slides/chips), applications, and difficulties of the technology in the area of skin biology will be presented.

Keratinocyte-Based Mechanistic Assays for Chemicals that Cause Contact Dermatitis

Anthony Gaspari, M.D.
Department of Dermatology
University of Rochester

Keratinocytes are known to be important participants in allergic contact dermatitis. This important cell type of the epidermis can produce a variety of cytokines, interact with Langerhans cells and epidermotrophic T-lymphocytes, and be induced to express adhesion molecules by cytokines produced by infiltrating inflammatory cells. Keratinocytes have been noted to express CD80-like molecules during inflammatory skin diseases, including contact dermatitis. We have demonstrated that the cell surface expression of CD80 is regulated by exposure to certain allergens and irritants in cultured human

keratinocytes. The upstream, untranslated DNA sequences (promoter/enhancer fragments) were cloned into luciferase-containing gene reporter constructs. Normal human cultured keratinocytes were transiently transfected, and basal and inducible CD80 gene expression was studied using the gene reporter constructs. Certain chemicals that induce contact dermatitis cause an increase in the expression of CD80 gene reporter fragments in cultured human keratinocytes. These data suggest that this is a viable strategy for developing a multi-endpoint keratinocyte adhesion/costimulatory molecule based-gene reporter assay to identify chemicals that cause contact dermatitis.

A Molecular Diagnostic Approach to Irritant or Allergic Patch Testing Using the DermPatch

Melissa Fitzgerald, Ph.D., Lawrence A. Rheins, Ph.D., Vera Morhenn, M.D., Stacey Humphrey and Nirmala Jayakumar
DermTech International, San Diego, CA

Differentiation between allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) reactions can be difficult by visual observations only of the degree of erythema in the skin. To objectively identify and grade the severity of skin reactions, we are developing a molecular diagnostic assay for distinguishing ACD from ICD based on mRNA profiles in the epidermis. The DermPatch is a non-invasive skin sampling technology that has potential for isolating sufficient quantities of mRNA for gene expression profiling. Key markers of ACD and ICD obtained from in vitro skin models or from biopsy samples will be reviewed, and compared with results obtained using the DermPatch.

Standardized Human Epidermal and Mucosal Tissue Models for the Screening of Potential Lead Compounds in Pharmaceutical Development

Bart De Wever, ir.
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SkinEthic Laboratories is a leading tissue engineering company specializing in the reconstruction of human epidermal and epithelial tissues for *in vitro* testing applications for the pharmaceutical, chemical and consumer product industry. Using its proprietary technology, the company has developed a semi-automatic tissue production process in totally defined cell culture conditions, allowing mass-production of human epidermal and epithelial tissue constructs with normal histology, functionality, and unmatched reproducibility.

The reconstituted epidermis is similar to *in vivo* human epidermis and is covered by a functional stratum corneum barrier. Many major European and US pharmaceutical, chemical, and consumer product companies use it routinely for testing the penetration, metabolism, skin irritation, or efficacy of their products. Due to the defined medium and the tissues long-term stability, the acute and chronic skin irritation test procedures allow detection of irritating compounds after 24 hours, but also of 'sub-clinical' adverse effects of cosmetic creams (occurring *in vivo* only in sensitive skin after 3 weeks) already after 72 hours of topic application onto SkinEthic epidermis, using interleukin-8 quantification assays. Additionally, by daily topical application for 6 days on reconstructed human epidermis of three different photo-types, the pigmenting or depigmenting effects of active agents or finished creams can be assessed visually and quantitatively.

The reconstituted human corneal epithelial tissues allow routine screening of all types of compounds for their ocular irritation potential, replacing favorably and completely the *in vivo* Draize ocular irritation test. The more recently developed buccal and vaginal mucosal tissues are used to test *in vitro* the activity or irritation potential of dental, oral or vaginal care products. Production of other human epithelial tissues (gingival, esophagus, nasal, pulmonary) is currently in development.

The characterization and development of these human tissue-engineered models, and their successful current use in industrial *in vitro* testing, will be presented.

In Vitro Skin Equivalent Models for Toxicity Testing

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In vitro models of differentiated human dermal epithelial tissues can be produced by culture at the air/liquid interface (ALI). These models possess in vivo-like characteristics and barrier properties which allow application of candidate therapeutic compounds as well as finished formulations in a more realistic manner compared to cultures submerged in media. Two such models, EpiDerm™ (composed of human keratinocytes) and MelanoDerm™ (composed of human keratinocytes and melanocytes) are described in the present work. A critical issue for successful utilization of ALI tissues in commercial safety screening and product development applications is reproducibility of the models. Therefore, a quality control procedure based on histological evaluation and a quantitative MTT viability assay has been designed to insure tissue reproducibility. Quantitative data compiled over the past 6 years demonstrate a high level of intra-lot and inter-lot reproducibility for EpiDerm™.

A survey of applications including irritation, phototoxicity, and corrosivity will be presented for EpiDerm. Applications of MelanoDerm for pigmentation studies will also be presented. Distinct MelanoDerm phototypes can be created depending on the source of melanocytes used to generate the tissue. As expected, tissue containing Black, Asian, or Caucasian melanocytes produce skin with pigmentation levels following the expected order, i.e. Black > Asian > Caucasian. In addition, the topical application of materials known to inhibit melanogenesis results in decreased tissue pigmentation, as evidenced by visual observation and a quantitative melanin assay. Finally, new tissue models including high-throughput formats, fibroblast-containing and Langerhans cell-containing tissues and new genomic applications will be presented.

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Neurotoxicology (Molecular Biomarkers, Transgenics and Imaging Technologies)

Session Chairs: Thomas Sobotka, Ph.D., US FDA and William Slikker, Jr., Ph.D., US FDA

Understanding the linkage of the environment to the genome represents a major challenge to the toxicologist. Within the nervous system, the multitude of cell types and regional functionality increase the difficulty of understanding signal transduction an order of magnitude above other less complex organs. New tools, including genomics, proteomics, transgenics and imaging are needed to unravel the complexity of the nervous system. The presenters in this symposium will address each of these issues and describe the progress of the application of these new or improved tools to study neurotoxicity. An understanding of, and the development of markers for, generic responses of the nervous system to neurotoxic exposures offer one avenue for screening for neurotoxic effects of broad classes of chemicals or chemical mixtures. The identification of sensitive, early molecular biomarkers of neurotoxicity including members of the family of cell adhesion molecules known as integrins that regulate cell migration and attachment to extracellular matrix and contacts with other cells, provide mechanistic data linking exposure with adverse effects and/or susceptibility. Gene-expression patterns, assessed by gene-array technologies (filter or microarray), proteomics, assessed by analytical mass spectrometry, and toxicant-induced cell-signaling, assessed by phospho-state-specific antibodies linked to gene transcription, offer the potential for detection and characterization of novel markers of neural injury associated with the above-mentioned neurotoxic responses. Developing a database of novel neural injury markers using these techniques can provide a molecular and cellular basis for interpreting and validating data obtained with emerging noninvasive imaging technologies. While techniques such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) provide important information on the localization of pharmacological agents and their pharmacodynamics and pharmacokinetics, new applications of MRI and PET provide the ability to noninvasively detect gene expression in higher organisms. The development of in vitro imaging techniques that employ trophic factor stimulated and differentiated rat pheochromocytoma (PC12) cells in culture can recapitulate early and later events in the process common to neuronal differentiation. These new approaches employ quantitative morphological methods to evaluate both pharmacological and toxicological effects. In so doing, a rapid, inexpensive, imaging-based approach to neurotoxicity assessment can be implemented with an associated reduction in animal usage.

Methamphetamine-induced Neurotoxicity: Lessons from Genetically Engineered Mice

Jean Lud Cadet, M.D.
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Development and Evaluation of *In Vitro* Imaging Techniques Used To Screen Agents That Affect Neuronal Differentiation

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Testing chemicals for potential developmental neurotoxicity is an issue of regulatory concern. Developmental vulnerability is determined by pharmacokinetic and pharmacodynamic differences between developing and adult animals. These pharmacokinetic and pharmacodynamic factors determine the target tissue dose and the limits of the window(s) of susceptibility. The focus of our recent work has been on the development of rapid and quantifiable alternative test methods that both recapitulate developmental processes and which are sensitive to known developmental neurotoxicants. Differentiation is the principal process that underlies the diversity of cell types in the nervous system and the first test system being developed and evaluated in our lab. We have developed imaging techniques

that employ use a rat pheochromocytoma (PC12) cell line that recapitulates early and later events in this process that are common to neuronal differentiation. This *in vitro* approach employs quantitative morphological methods can be used in other culture systems of primary cells and slices. Furthermore, this methodology and can be extrapolated to similar measures that can be performed on specifically stained cells in histological sections from *in vivo* exposed animals. The evaluation of this *in vitro* assay system has used known trophic factors (NGF and bFGF) as positive controls for stimulation of neuronal differentiation and used pharmacological agents that block these signaling systems leading to reduced neuronal differentiation. Recently, this assay system has expanded into a new phase of evaluation with the examination of known and suspected developmental neurotoxicants (methylmercury, mercuric chloride, lead, polychlorinated biphenyl mixtures, and chlorpyrifos). These compounds can have differential effects on neurite outgrowth (*i.e.*, differentiation), with some affecting initiation events and some affecting later events, but all lead to alteration in neuronal morphology. These compounds also have unique concentration responses including some that were not unimodal. All of these compounds had effects on some aspect of differentiation in the absence of overt cytotoxicity. (*This abstract does not reflect USEPA policy*).

Potential Applications of Noninvasive Imaging in Toxicology Research

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The impact of biomedical imaging has been unquestionable. Techniques such as Magnetic Resonance Imaging (MRI), Computed Tomography scanning (CT) and Ultrasound (USG) have become vital to the identification and diagnoses of many disease states and pathologies. The ability to visualize such altered states in a noninvasive manner and in real-time have allowed rapid diagnosis and subsequent treatment for the betterment of public health and safety. Additionally, these techniques allow monitoring of the treatment effect on the targeted pathology. Other techniques such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) provide important information on the localization of pharmacological agents and their pharmacodynamics and pharmacokinetics. New applications of MRI and PET provide the ability to noninvasively detect gene expression in higher organisms. Much of this information cannot be obtained using conventional pathology or related approaches. The transfer of this technology to toxicological assessment and the regulatory arena has been slow. This has been due in part to the cost of the instrumentation, but more importantly, the concern relating to interpretation of the images obtained. In order to verify this data, conventional practices must complement imaging data until an appropriate "comfort" level (scientific consensus?) is reached. An overview of the different approaches and their potential application to toxicology will be presented. Methodologies to be presented include MRI, CT, and PET and some of their variations. Using imaging approaches in toxicology will require the toxicologist to develop a different approach than is presently being applied. The ultimate result will be faster, more conclusive studies that will reduce the required number of animals and may lead to noninvasive clinical endpoints.

Molecular Strategies for Neurotoxicity Screening: Moving Beyond the One Compound, One Mechanism Approach

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The molecular and cellular targets of neurotoxic insult are diverse and unpredictable, owing to the extreme molecular and cellular heterogeneity of the adult and developing nervous system. This neurobiological complexity constitutes the central dilemma for contemporary neurotoxicology, because any attempt to screen for neurotoxic effects associated with environmental, occupational or pharmaceutical exposures must overcome this obstacle. Current funding practices tend to favor "mechanistic" evaluations of specific

chemicals/drugs/mixtures presumed to be potentially toxic to the human nervous system (e.g. metals, cholinesterase inhibitors, amphetamines, & PCBs). While such research may be invaluable for understanding neurotoxicity mechanisms associated with exposure to a particular compound or even a class of compounds, there is no reason to believe that such knowledge can be broadly applied for screening the thousands of existing and future chemicals that may pose a neurotoxic threat to humans. Indeed, given the complexity of the nervous system at all levels, it seems likely that a one compound, one mechanism of action may be the rule for neurotoxic agents, not the exception. This dictates the need for screening approaches that encompass all potential nervous system targets and mechanisms in order to provide the most effective defense against human exposures to neurotoxic agents. An understanding of, and the development of markers for, generic responses of the nervous system to neurotoxic exposures offer one avenue for screening for neurotoxic effects of broad classes of chemicals or chemical mixtures. Such responses include: glial activation (micro- and astroglial), proteolysis, neuroinflammation and generation of reactive oxygen species linked to cellular damage. Gene-expression patterns, assessed by gene-array technologies (filter or microarray), proteomics, assessed by analytical mass spectrometry, and toxicant-induced cell-signaling, assessed by phospho-state-specific antibodies linked to gene transcription, offer the potential for detection and characterization of novel markers of neural injury associated with the above-mentioned neurotoxic responses. Developing a data base of novel neural injury markers using these techniques can provide a molecular and cellular basis for interpreting and validating data obtained with emerging noninvasive imaging technologies. In so doing, a rapid, inexpensive, imaging-based approach to neurotoxicity assessment can be implemented with an associated reduction in animal usage.

Development of Integrin Expression as a Molecular Biomarker for Early, Sensitive Detection of Neurotoxicity

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In the past toxicologists have relied largely on evidence of overt toxicity (either functionally or morphologically) to acute exposures to assess toxicity. In toxicology today determining the consequences of low level and/or chronic exposure(s) is gaining new priority. That is levels of exposure that produce subtle changes that result in delayed effects or have greater consequences in susceptible populations (e.g. the young or aged). One of the major impediments to studying these kinds of effects is the lack of methods for measuring these subtle changes. The identification of sensitive, early molecular biomarkers of neurotoxicity will be of great benefit. Biomarkers that provide mechanistic data linking exposure with adverse effects and/or susceptibility and provide data that can reduce some of the uncertainty factors in risk assessment. For example, a well-defined biomarker could address issues of extrapolation from *in vitro* studies to *in vivo* consequences or provide an efficient method for identifying areas of susceptibility in dose response and/or exposure times.

Necessary to normal CNS development, function and recovery after insult are cell processes involving cell migration, attachment to extracellular matrix (ECM) and contacts with other cells, both like and unlike. Members of the family of cell adhesion molecules known as integrins, have been shown to play important roles in each of these processes. As dimeric trans-membrane proteins, they serve to transduce signals bi-directionally between extra-cellular components such as extra-cellular matrix and other cell adhesion proteins and intracellular components such as transduction pathway proteins (Clark and Brugge, 1995) and the cytoskeleton. Formed from heterodimers of α and β protein subunits, the composition of the heterodimeric receptor is determined by tissue and cell type as well as specific cellular responses (Huhtala et al., 1995). The combination of participation in a number of critical nervous system processes with the potential for tissue and temporal specificity make the expression of integrins ideal candidates as molecular biomarkers. *(This abstract does not reflect USEPA policy)*

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Friday, December 1

Role of Transgenics and Toxicogenomics in the Development of Alternative Toxicity Tests

Session Chairs: Jerry Heindel, Ph.D., NIEHS and John Frazier, Ph.D., US Air Force

Introduction

Jerry Heindel, Ph.D.
National Institute of Environmental Health Sciences

The Use of Transgenics in Carcinogenicity Testing: An Update

Denise Robinson, Ph.D.
International Life Sciences Institute

Toxicogenomics and Toxicity Testing

Ben Van Houten, Ph.D.
National Institute of Environmental Health Sciences

Mammalian Gene-Trapping with the Beta-Lactamase Reporter – Applications to Toxicogenomics

Gregor Zlokarnik, Ph.D.
Aurora Biosciences Corporation
San Diego, CA

The beta-lactamase reporter assay provides an enzymatically amplified fluorescence readout of gene expression in live mammalian cells. It is compatible with flow cytometry and enables ultra-fast functional selection of cells with reporter-trapped stimulus responsive genes. Tagged genes are identified by RACE and selected cell lines can be used as sensors for the stimulus. The application of this technology to elucidating the actions of aromatic hydrocarbon toxins and their polychlorinated derivatives on gene expression will be discussed.

Non-invasive Real-time Toxicodynamics

Karen Steinmetz, Ph.D.
Xenogen Corporation
Alameda, CA

Role of Imaging in the Development of Alternative Toxicity Tests

Session Chairs: Doug Kawahara, Ph.D. (Xenogen) and Karen Hamernik, Ph.D. (EPA)

Nanostructures and Toxicology

Martin Philbert, Ph.D.
University of Michigan

MRI and Biophotonic Imaging

Brian Ross, Ph.D.
University of Michigan

Two Photon Microscopy: Application to Toxicology

Peter So, Ph.D.
Massachusetts Institute of Technology

Concluding Discussion and Remarks

Douglas Kawahara
Xenogen Corporation
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Meeting Summation

Harry Salem, Ph.D.
US Army Edgewood Chemical Biological Center

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Biographical Sketches

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B.S. Biology, 1982, Belmont Abbey College, Belmont, NC

August- November, 2000 Acting Branch Chief of Cellular and Molecular Toxicology Branch of Neurotoxicology Division
1997- Present GS-13 Research Biologist in Cellular and Molecular Neurotoxicology Branch, USEPA with special attention to development of mechanistically-based markers of developmental neurotoxicology to be used *in vitro* and *in vivo* test systems.
1995- 1997 GS-12 Research Biologist in Cellular and Molecular Neurotoxicology Branch, USEPA with special attention on alterations in neurotrophic factor interactions and related signal transduction proteins as indices of developmental neurotoxicology.
1994- Research Scientist at ManTech Environmental a contractor for USEPA with a research program encompassing cellular and molecular markers of developmental neurotoxicity with special attention on neurotrophic factor interactions and related signal transduction proteins.
1992-94 Project Scientist at ManTech Environmental a contractor for USEPA working under the supervision of Maria del Valle-Torres. Project includes neurotoxicant-induced changes in neurotransmitter systems with special attention on development of the central nervous system and expression of neurotrophic factor and their receptors.
1990-92 Senior Scientist at ManTech Environmental a contractor for USEPA working under the supervision of Mary Gilbert, Ph.D. Project included neurotoxicant-induced changes in neurotypic and gliotypic markers of peripheral and central nervous system damage.

RESEARCH INTERESTS:
* *Cellular and molecular markers* of neurotoxicant-induced damage with particular attention to cortical development.
* Trophic factor involvement in development, degeneration and compensation of the nervous system.
* Cholinergic markers of the basal forebrain and their cortical projections.
* Quantitation of morphological changes in the nervous system using computer assisted image analysis.

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Mr. Brown is a board-certified toxicologist in the Health Sciences Branch, Division of Life Sciences, Office of Science and Technology, CDRH/FDA. His research interests at FDA focus on the development and validation of new methodologies to assess the risk posed by exposure to compounds released from medical devices. In addition to developing

new risk assessment approaches, Mr. Brown conducts risk/safety assessments in support of both pre-and post-market regulatory decision-making activities in CDRH. Mr. Brown also represents the FDA and the United States on ISO technical committee working groups involved with the development of consensus standards for the biological evaluation of medical devices. Prior to joining FDA, Mr. Brown held a position at the ILSI Risk Science Institute.

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Wiley A. Chambers, MD, is the Deputy Director of the Division of Anti-Inflammatory, Analgesic and Ophthalmologic Drug Products at the Food and Drug Administration (FDA). Dr. Chambers completed medical school and a residency in Ophthalmology at the George Washington University School of Medical and Health Sciences in Washington, DC. He joined the FDA in 1987, as a primary reviewer for ophthalmic drug products and in 1990 became the Supervisory Medical Officer for Ophthalmologic Drug Products. In this capacity, Dr. Chambers has supervisory responsibility for the clinical review of ophthalmologic drug products submitted to the FDA for study and potential approval. Additionally, Dr. Chambers is the recipient of numerous Public Health Service, FDA and Center for Drug Evaluation and Research awards for his work with the FDA.

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Mr. Nicholas J. DelRaso is currently a research toxicologist in the Operational Toxicology Branch of the Air Force Research Laboratory, in the Human Effectiveness Directorate at Wright-Patterson AFB, OH. He received a Bachelor of Arts in Microbiology/Zoology in 1981 from Ohio Wesleyan University and a Master of Science in Microbiology from Wright State University in 1987. His research has concentrated on developing *in vitro* methods to assess toxic effects of Air Force chemicals on mammalian cell systems. Recent research interests have focused on *in vitro/in vivo* extrapolation using cadmium as a model compound to predict dose/time dependency of acute hepatotoxicity, and development of a deployable cell-based assay system for predicting potential hazards in Air Force operational areas.

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Bart De Wever, ir., is director of Business Development at SkinEthic Laboratories, Nice, France since 1998. As a biochemical engineer, he has been actively involved for many years in the development of in vitro technology: From 1993 to 1997, he was a consultant for Advanced Tissue Sciences, La Jolla, CA, USA, and responsible for their Skin$^{2®}$ In Vitro Laboratory Technology business in Europe. Before, he held a research position in the in vitro dermatology department at Janssen Pharmaceuticals in Belgium. He is an active member of several toxicological societies and has been author and co-author of many in vitro skin technology papers.

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Current Position and Responsibilities:
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- expert toxicologist - alternatives (manage Unilever's alternatives research programme), animal testing issues, test guidelines, validation and regulatory acceptance of new tests
- manager of SEAC Toxicology's external research programme/budget
- project manager - Unilever safety evaluation and investigative toxicology research projects

Qualifications:
Ph.D. (Biochemical Toxicology) - University of Nottingham Medical School, UK - 1991
M.Sc. (Toxicology) - School of Biochemistry, University of Birmingham, UK - 1988
B.Sc. (Hons) Biochemistry: Class I - Department of Biochemistry, University of Leeds, UK - 1987

Previous Positions:
Scientific Officer (Toxicologist; Head of Section), European Commission (ECVAM, Joint Research Centre, Ispra, Italy) - 1994-1998
Scientific Liaison Officer, FRAME (Nottingham, UK) & Visiting Research Fellow, University of Nottingham Medical School - 1991-1994

Expertise:
Alternative (*in vitro*) testing methods; eye and skin irritation/corrosion; metabolism-mediated toxicity; regulatory toxicology; testing guidelines and strategies; validation

Awarded the first European Commission Joint Research Centre's Young Scientists Prize in June 2000, for the contribution made to the validation and regulatory acceptance of alternative test methods

Publications:
25 full, peer-reviewed, papers; 30 invited articles/chapters; 17 reports (12 published in journals); 20 abstracts; educational resource materials; numerous papers in conference proceedings

Other Information:
Member of: Institute of Biology (CBiol, MIBiol); British Toxicology Society; *In Vitro* Toxicology Society (IVTS - Secretary, Executive Committee); European Society for Toxicology *In Vitro* (ESTIV); Editorial Board of the scientific journal *Alternatives to Laboratory Animals* (ATLA); UK DoH OECD Shadow Group (testing guidelines); Management Team for the ECVAM Skin Corrosivity Validation Study (Chairman);

Management Team for the ECVAM Skin Irritation Prevalidation Study (Coordinator); COLIPA's Steering Committee for Alternatives to Animal Testing (SCAAT); COLIPA Eye Irritation Task Force; COLIPA Skin Tolerance Task Force; FRAME Toxicity Committee; Management Team for the ECVAM Eye Irritation (Reference Standards) Study; ECVAM Validation and Regulatory Acceptance Task Force; ECVAM Skin Irritation Task Force; several OECD steering committees/expert groups (skin penetration guidelines, acute toxicity tests, etc.); ICCVAM Peer Review Panel for Corrositex™; invited expert for ICCVAM review of *in vitro* tests for predicting acute systemic toxicity (Co-Chair); Scientific Committee for INVITOX Workshops; Scientific Committee for AMFEP Validation Programme on Enzyme Concentrates

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Dr. Fitzgerald joined DermTech International in 1999 as the Director of Business Development. Currently, she is responsible for research and corporate development for the DermPatch, the company's [patent pending technology for non-invasive isolation and detection of biomarkers from the skin. From 1995 to 1999, Dr. Fitzgerald was a Scientist at Dura Pharmaceuticals, developing dry powder formulations of protein and peptide therapeutics for non-invasive delivery with the Spiros inhaler. Her experience includes designing pre-clinical studies with novel pulmonary inhalation systems, and preparing formulations for toxicological and human clinical studies. At Dura, she was a member of the strategic business development team to evaluate new opportunities for drug delivery of macromolecules. Dr. Fitzgerald's previous experience included postdoctoral work in X-ray crystallography and protein engineering at the Scripps Research Institute. She obtained a Ph.D. in Physical Chemistry from Virginia Commonwealth University, the Diplome D'Etudes Approfondies in Organic Chemistry from the l'Universite Louis Pasteur in Strasbourg, France, and a B.S. in Chemistry from Loyola College in Maryland.

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Post doctoral position at Dept. of Neurochemistry and Neurotoxicology, Stockholm University, 1993-1994
Project leader at Dept. of Neurochemistry and Neurotoxicology, Stockholm University, 1994-1998

Author of about 25 scientific articles and reports and about 30 abstracts for scientific meetings. Teacher in cell toxicology and neurochemistry, supervisor of three Ph.D. students and former supervisor of ten M.Sc. students. Invited speaker and consulted as expert in the field of *in vitro* neurotoxicology at several occasions. Member European Soc. of Toxicology *In Vitro*, Scandinavian Soc. of Cell Toxicology (secretary) and Swedish Soc. of Toxicology. Board member of the Swedish Fund for Research without Animal Experiments.

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Dr. Gaspari is currently Associate Professor of Oncology in the Cancer Center, University of Rochester, and Associate Professor of Dermatology in the Department of Dermatology, University of Rochester. He received a B.A. in Biology from Temple University, Philadelphia in 1976 and a M.D. from Jefferson Medical College, Philadelphia, in 1981.

His current research interests include defining, at the molecular level, the direct effects of allergens and irritants on epidermal antigen presenting cells.

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Dr. Hayden received his B.S. degree in Chemistry from the University of Maryland in 1984 and his Ph.D. in Chemistry from Clarkson University, Potsdam, NY in 1991. His graduate research in molecular toxicology was conducted in the laboratory of Dr. James Stevens at the W. Alton Jones Cell Science Center in Lake Placid, NY. Dr. Hayden also received post-doctoral training in free radical chemistry and toxicology as a PRAT fellow in the laboratory of Dr. Colin F. Chignell at NIEHS. He then took a position with the Gillette Company, where he investigated mechanisms of skin irritation and toxicity in in vitro systems. He joined MatTek Corporation 1999, where he is involved in development of in vitro tissue models and assay methods for use in pharmacologic and toxicologic applications.

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has been a scientific program administrator in the Division of Extramural Research and Training at NIEHS. He is responsible for developing a research portfolio in the areas of reproductive toxicology, developmental toxicology, endocrine toxicology, immunotoxicology and alternative methods and models that will address the major environmental health problems of the American people. This is accomplished through program announcements, requests for applications(RFA), workshops/symposia and direct contact with the scientific community.

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Dr. Jester received his graduate training in Experimental Pathology from the Department of Pathology, University of Southern California, Los Angeles, California. He went on to post-doctoral studies in Experimental Ocular Pathology at the Estelle Doheny Eye Foundation, USC Medical Center, and later at the National Institutes of Health, National Eye Institute, Bethesda, Maryland. Dr. Jester has held previous positions as Assistant Professor of Ophthalmology and Pathology at USC Medical Center from 1983-1986, and Associate Professor of Ophthalmology and Pathology at Georgetown University Medical Center, Washington, D.C., 1986-1991. Dr. Jester is currently Professor of Ophthalmology at the University of Texas Southwestern Medical Center at Dallas, Dallas, Texas. Dr. Jester's research focuses on studies of corneal wound healing and refractive surgery and has published over 120 papers in peer-reviewed journals in this field. Two National Eye Institute, National Institutes of Health research grants funds his research into studies of the cellular and molecular mechanisms of TGFβ mediated corneal keratocyte activation and the role of keratocyte crystallin protein expression in the maintenance of corneal transparency. For past 6 years Dr. Jester has worked closely with Dr. James K. Maurer of the Procter & Gamble Company on the confocal microscopic characterization of ocular irritation in the rabbit using the LVET test. Based on findings in these studies, he and Dr. Maurer have proposed that the basis of ocular irritation critically involves the extent of corneal injury. Recent published data suggest that extent of injury may also be used in the development of mechanistically based alternative models of eye irritation. Currently, Dr. Jester is supported by the Center for Alternatives to Animal Testing in Baltimore, Maryland, the Lion's Eye Bank Foundation, Houston, Texas and the Eye Bank Association of America to develop extend-life human corneal cells using human Telomerase Reverse Transcriptase and produce human corneal constructs using these cells to test whether extent of injury can be used as a basis for the further development of alternative tests.

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Dr. Jett received his B.S. degree in Biology from Hampton University in 1980, his M.S. in Zoology in 1983, and his Ph.D. in Toxicology in 1992 from the University of Maryland. He then trained as a postdoctoral fellow and served as a Research Associate at Johns Hopkins where he was appointed to the Faculty in Toxicological Sciences in 1996. The focus of research in his laboratory is in the field of neurotoxicology, with special emphasis on the effects of pesticides and metals.

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Dr. Jones received his B.A. degree in Biochemistry from the University of Kansas in 1988. He then worked as a Research Assistant in the Department of Toxicology at the University of Kansas Medical Center, where, in 1993, he entered graduate school. He received his Ph.D. in Toxicology in 1996. In 1995, Dr. Jones began work as a Scientist at Mary Kay Holding Corp. in the Product Safety Department, focusing on *in vitro* safety testing methods. Dr. Jones has since worked as Manager, Toxicological and Clinical Sciences at Cosmair and is currently Program Manager heading up the Cell Biology & *In Vitro* Toxicology group at Avon Products, Inc. Current research interests include molecular biology-based techniques for assessing safety and efficacy of personal care ingredients and products at Avon.

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Dr. Kawahara received a M.S. in medical microbiology and Ph.D. in cellular immunology from the University of Rochester School of Medicine and Dentistry. From 1981 to 1985, he was a postdoctoral fellow and lecturer at the University of California, Los Angeles. From 1985 to 1990, Dr. Kawahara served as Director of Research in autoimmune diabetes at Children's Hospital of Orange County, California. In 1990, he received a M.B.A. in Finance from California State University, Long Beach. Dr. Kawahara is a member of the American Association of Immunologists, the Clinical Immunology Society, and Sigma Xi.

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Dr. Lein received her B.S. degree in Biology from Cornell University in 1981 and M.S. in Environmental Health from East Tennessee State University in 1983. In 1990, she received her Ph.D. in Pharmacology and Toxicology from the State University of New York at Buffalo School of Medicine. She did additional training in molecular immunology as a postdoctoral fellow at Roswell Park Cancer Institute in Buffalo, NY and worked as an environmental health analyst for the international consulting firm, Dames and Moore. In 1993 she was appointed to assistant professor in the Biology Department of Canisius College in Buffalo, NY. She joined the Johns Hopkins University faculty as Assistant Professor of Toxicological Sciences in 1999.

Research in her laboratory is focused on elucidating the cellular and molecular mechanism(s) by which neurons develop their characteristic morphology and the mechanisms by which developmental neurotoxicants disrupt these developmental proceses.

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Dr. Lester is Team Leader of the Neural and Cellular Pharmacology Research Team, Division of Applied Pharmacology Research, Center for Drug Evaluation and Research, Food & Drug Administration, Laurel, Maryland. He received his B.Sc. (Hons.) degree from the University of New South Wales, Australia, a M.Sc. from the Hebrew University of Jerusalem, Israel, and a Ph.D. from Northwestern University, in 1984. He completed postdoctoral fellowships at the Department of Pharmacology, Harvard University Medical School (1983-1985), and the Department of Membrane Research, Weizmann Institute of Science (1985-1986). He was a Staff Scientist at the Department of Membrane Research, Weizmann Institute, from 1986-1990. Dr. Lester was a Visiting Scientist in the National Institute of Neurological Disorders and Stroke, NIH, from 1990-1993. He joined the FDA in 1993. Dr. Lester has over 70 scientific publications and has edited three books. He is currently editing a fourth book on Neurotoxicity. He is co-chair of the Neurotoxicity Assessment Committee of CDER, on the Board of Directors of the Society for Nuclear Imaging in Drug Development, and the Science Policy Committee of FASEB.

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Michael Anthony Major holds an undergraduate degree in biology (1972) and a doctorate in physical/organic chemistry (1978) from the University of Southern Mississippi. He is a member of the American Chemical society and has authored over 50 journal articles technical reports and papers. Doctor Major was a graduate of the Army Chemical Officers Advanced Course and the Army Command and General Staff College. He served as an officer on the headquarters staff of the 28th Infantry Division as the NBC Center Director and later as the Assistant Division Chemical Officer. His work as a Civilian Army Chemist has been in the areas of prediction of the environmental fate and toxicity of military pollutants. He was the co-developer of a cleanup process that utilizes changes in redox potential to covalently bind nitroaromatic pollutants to soil and is the USACHPPM patent applicant for a process to chemically convert TNT to quinonic compounds that undergo rapid microbial degradation. His most recent work with chemical agents include predictions of the viability of pathways of uptake of chemical warfare residues from soil and prediction of the toxicity of the products generated during the Alternative Technology Destruction of the chemical agents HD and VX.

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Dr. Mershon is a veterinarian and retired U.S. Army civil servant, now employed as an SAIC Biomedical Analyst. He reviews cancer research reports submitted to the Congressionally Directed Medical Research Program administered by the U.S. Army. Prior efforts involved data collection and interpretation, as represented in his poster. However, Dr. Mershon has a long history of applying alternative toxicological methods. During 31 years prior to his retirement from the U.S. Army Medical Research Institute of Chemical Defense, APG, MD, he found unusual

ways to spare animals. In 1960, he used banana skins (in lieu of animals backs) to assess possibly caustic chemicals. During the 70's he used steel washers and microcentrifuge tubes to minimize testing of skin decontaminants on rabbits. In the 80's he modified a chemical agent detector for use as an "artificial animal". He used it, and M-8 chemical agent detector paper, to screen Scotchgard-like skin protectant coatings for use by persons at risk of exposure to toxic chemical agents. In the early 90's, he was the first scientist to use artificial human skin for detection of vesicant agent damage.

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Dr. James P. O'Callaghan is Head of the Molecular Neurotoxicology Laboratory in the Toxicology and Molecular Biology Branch of the Health Effects Laboratory Division at the Centers for Disease Control and Prevention, NIOSH. His research group investigates the molecular and cellular basis of astrogliosis, a dominant response of the central nervous system to chemical- and disease-induced injury. Prior to joining CDC-NIOSH, Dr. O'Callaghan served as the Senior Science Adviser to the Neurotoxicology Division of the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency (EPA). At the EPA and CDC-NIOSH, Dr. O'Callaghan has conducted extensive research on the neurotoxicity profiles of many types of chemicals. He also has examined the neurotoxic effects of drugs of abuse under sponsorship of the National Institute on Drug Abuse (NIDA). Dr. O'Callaghan has co-authored over 100 papers in the area of Neurotoxicology and his research findings have been presented by invitation at numerous national and international conferences. In 1992 he was awarded the EPA's and the Society of Toxicology's Science Achievement Award for his work in developing and validating a bioassay for neurotoxicity. Because of Dr. O'Callaghan's expertise in the area of neurotoxicity, he has worked as a consultant for a number of public institutions, including the U.S. FDA and the NIH. Dr. O'Callaghan also holds adjunct appointments at the New York University School of Medicine and The Laboratory of Molecular and Cellular Neuroscience at The Rockefeller University. He is an Associate Editor of Neurotoxicology and Teratology as well as a member of other editorial boards.

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Joseph J. Pancrazio earned a B.S. degree in electrical engineering from the University of Illinois, Urbana, in 1984, a M.S. degree in biomedical engineering from the University of Virginia, Charlottesville, in 1988, and a PhD degree in biomedical engineering from the University of Virginia in 1990. After postdoctoral training in pharmacology as a recipient of a National Research Service Award, he received a joint appointment in the departments of anesthesiology and biomedical engineering as an assistant professor of research the University of Virginia in 1991. While conducting research into molecular mechanisms of anesthetic action, he acted as thesis advisor for three biomedical engineering graduate students and developed graduate courses in the

departments of biomedical engineering and pharmacology. In 1996, he joined Science Applications International Corporation as a senior electrophysiologist and, in 1997, joined Georgetown University department of biochemistry and molecular biology as an assistant professor of research working at the Naval Research Laboratory. In 1998, he joined the Naval Research Laboratory in the Biosensors and Biomaterials group as a Material Research Engineer. He presently leads externally-supported programs in portable cell-based sensor design and gene expression analysis for toxicology. His interests include bioelectrical systems, in vitro assay development, bioinformatics, and biological system modeling.

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Dr. Platteborze obtained his Ph.D. in biochemistry/molecular biology in 1995 from the University South Carolina. From there he reentered the military as an O-3 and his initial tour was at USAMRICD (1996-99). There he was PI investigating both nerve agent poisoning and mustard gas injury. Work centered around establishing recombinant protein expression systems for human butyrylcholinesterase as well as generating site-directed mutants of BuChE that could hydroylze OPs. In regards to sulfur mustard research, differential display PCR was established and DNA arrays were screened to begin to get an understanding of the transcriptional effects of SM at early time points as well as this project. Dr. Platteborze is currently stationed at USAMRIID (1999-2000) and works in the Virology division. His work emphasis is on vaccine development of infectious diseases.

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Prabhati Ray, Ph. D., is a Research Chemist and the Chief, Section on Molecular Biology, Department of Biology at the Walter Reed Army Institute of Research (WRAIR), Washington, D. C. Dr. Ray's laboratory is engaged in studies on the mechanisms of action of chemical and biological compounds and development of antidotes utilizing in vitro cell culture models. Dr. Ray's current research interests include molecular characterization of proteases stimulated by the vesicating compound sulfur mustard and studying the mechanism of botulinum toxin A effects on neuroexocytosis.

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Dr. Robinson is Executive Director of the International Life Sciences Institute's Health and Environmental Sciences Institute (HESI) and has served as HESI's director since 1992. In this capacity, she provides overall technical guidance and support for HESI's scientific programs as well as direction for the development of new scientific initiatives in the areas of carcinogenesis, reproductive toxicology, immunotoxicity, testing methodologies, epidemiology, exposure assessment and risk assessment. She serves as the primary public representative for the institute, and coordinates the development of written materials on HESI programs for public dissemination.

Dr. Robinson joined ILSI in 1991 as Senior Scientist in the ILSI Risk Science Institute, after holding post-doctoral positions at Georgetown University in the Toxicology and Applied Pharmacokinetics program and at the National Institutes of Health. She has also had several years of toxicological consulting experience with Mitre Corporation and the Center for Environmental Health and Human Toxicology. She attended George Washington University and Georgetown University where she received her Ph.D. in pharmacology. She received a B.S. with honors in biology from the University of Cincinnati. Dr. Robinson is the author of a number of publications in the areas of risk assessment, toxicology, pharmacokinetic modeling, clinical pharmacokinetics, and neuropharmacology.

Dr. Robinson is a member of the American College of Toxicology, the Society of Risk Analysis, the Society of Toxicology and other scientific associations. Her expertise lies in the areas of methodologies for the assessment of risk to human health, biological mechanisms of toxicity, water safety, food safety and risk assessment legislation, design and interpretation of data from toxicity studies, and the use of pharmacokinetic models for interspecies extrapolation and risk assessment.

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Laurie Roszell received her Ph.D. from the University of Maryland Program in Toxicology in 1995. Her research at the

University of Maryland, and later at Mississippi State University, focused on the effects of xenobiotics on immune function in fish. In 1998, she began working for the US Army at the Center for Health Promotion and Preventive Medicine (CHPPM). Her work for the CHPPM includes providing professional guidance addressing potential toxicological effects of chemicals of military interest, deriving values representing safe levels for acute and chronic exposures, and utilizing EPA-approved methodology as well as QSAR and surrogate compounds. Her research focuses on the use of immortal cell lines to assess *in vitro* toxicity of individual and mixtures of compounds of military interest.

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Dr. Joyce Royland received a BS in Biology and a MS in Physiology from San Diego State University and a Ph.D. in Pharmacology/Toxicology from Oregon State University. Following graduate school she completed a postdoctoral fellowship at the West Virginia University Medical School on the effects of undernutrition on myelin gene expression during development. A Fellowship from the National Parkinson's Foundation took her to The Parkinson's Institute, a non profit research organization, where her research involved nitric oxide induction as a possible mechanism of neurodegeneration in Parkinson's Disease. Dr. Royland is now a member of the Neurotoxicology division of the US Environmental Protection Agency. Her research encompasses the development of mechanistic models of neurodegeneration. Currently she is investigating the potential use of the integrin family of cell adhesion molecules as biomarkers of effect after neurotoxicant exposure.

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Dr. Sassaman is the Director of the Division of Extramural Research and Training, National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health. She received a B.S. in Chemistry (with highest honors) from Auburn University and a Ph.D. in Microbiology-Immunology from Duke University. She continued her training in the Departments of Biochemistry and Medicine (Cardiology Division) at Duke Medical School and was a Research Assistant at that institution. She began her career in the Federal government as a chemist in the Bureau of Biologics, Food and Drug Administration, moving to the Blood Diseases Branch, Division of Blood Diseases and Resources, National Heart, Lung and Blood Institute, National Institutes of Health, in 1976.

As Chief, Blood Diseases Branch, Dr. Sassaman was instrumental in the development of national and international programs in thrombosis and hemostasis and received the NIH Director's Award for these efforts. She convened numerous workshops and conferences to develop research areas, including those which brought together biomedical scientists and bioengineers, and was on the steering committees for consensus development/assessment conferences related to such topics as thrombolytic therapy and bone marrow transplant registries.

She joined the staff of the NIEHS in her current capacity in 1986, and has led the

development of the extramural program of the Institute through a period of significant growth in terms of dollars and personnel as well as program diversity. Under her guidance, the Institute has taken a leadership role in the NIH in areas such as environmental justice, children's health and community-based prevention and intervention programs. She also led the Institute's establishment of two new programs created by the Superfund Amendments and Reauthorization Act of 1986 (SARA) the Superfund Basic Research Program and the Hazardous Waster Worker Training Program. Her Division has developed innovative trans-NIH and interagency programs and has worked collaboratively with other agencies in management of Congressionally-mandated initiatives such as the EMF RAPID program and the Department of Energy (DOE) program for training of clean-up workers at DOE facilities. She serves on a number of NIH-wide committees involved in policy and program development and received a second NIH Director's award for her work in support of career development of women scientists. Her NIEHS responsibilities include oversight and management of the Institute's peer review, grants administration, and research contracts administration activities. She interacts with scientists, science administrators, and policy-makers throughout the country and is the spokesperson regarding policy and priorities related to the extramural program of the Institute.

In 1999, Dr. Sassaman received a third NIH Director's Award for establishment of a national Interagency Children's Environmental Health Initiative.

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NeuroToxicology, 1994-present and *Toxicological Sciences*, 1999-present. Reviewer for NIH, HEI, EPA, ATSDR and NIEHS developmental and neurotoxicology grants and manuscripts. Research interests include developmental pharmacology, transplacental pharmacokinetics, neurotoxicology and risk assessment; mechanisms of toxicity and approaches to neuroprotection for substituted amphetamines, excitotoxicants and metabolic inhibitors. He has authored or co-authored over 235 peer-reviewed research articles and book chapters and co-edited the first comprehensive text on Developmental Neurotoxicology. Teaching Activities: Dr. Slikker has advised over ten doctoral and five postdoctoral students.

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A native of Baltimore, Maryland, Thomas J. Sobotka graduated from Loyola College with a B.S. in 1964. Under a graduate fellowship in the Department of Pharmacology, Loyola Stritch School of Medicine, Chicago, Illinois, he received his M.S. in 1967 and his Ph.D. in 1969. Since completion of his graduate studies, he has been employed by the Food and Drug Administration in the Center for Food Safety and Applied Nutrition, as a supervisory research pharmacologist. Dr. Sobotka established and continues to maintain an active neurotoxicology research program. The ongoing research of his team focuses on a variety of issues related to the detection and characterization of neurotoxic hazard for food related chemicals. He has authored/co-authored more than 60 scientific publications and book chapters in the area of behavioral and neurotoxicology and has made numerous presentations at various national and international conferences. Dr. Sobotka has also been routinely involved in various regulatory related activities including the review and evaluation of food additive, drug and medical device petitions, and the formulation of the neurotoxicity section of the FDA's recently revised guidelines for toxicological testing of food and color additives (Redbook). He has served on various workgroups and committees including the Interagency Committee on Learning Disabilities, the OECD working group on neurotoxicity and developmental

neurotoxicity, the Interagency Committee on Neurotoxicology of which he is currently the co-chair, the National Advisory Committee for Acute Exposure Guideline Levels, and the ILSI Working Group on Direct Dosing of Neonatal Animals in Toxicity Testing. Dr. Sobotka is also past-president of the Association of Government Toxicologists.

Karen Steinmetz, PhD.

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1981 - University of California, Davis - Advanced to Candidacy of Ph.D.

RECENT POSITIONS HELD
1992 to Present Associate Director, Human Safety Department, Human & Environmental Safety Division, The Procter & Gamble Company
1990 - 1992 Section Head, Clinical Pathology Section, Human & Environmental Safety Division, The Procter & Gamble Company

RESEARCH INTERESTS
(1) Toxicology, particularly the development, validation and acceptance of alternative methods, (2) endocrine mediate ovarian tumors in mice, (3) comparative pathology, specifically oncology, (4) spontaneous liver foci in rats, and (4) radiobiology.

AWARDS AND HONORS
Regent Award in Veterinary Medicine, University of California, Davis, 1968
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- Ocular toxicology, with emphasis on the corneal epithelium and endothelium and refinement of animal and in vitro methods. 29 publications on the related subjects of

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Received a Doctor of Veterinary Medicine (D.V.M.) degree, 1971, from Michigan State University, East Lansing, Michigan. Practiced clinical veterinary medicine through 1988 in Michigan. Active member of several professional and community organizations including 12 years on the Board of Trustees, Oaklawn Hospital, an acute care community hospital. Health care policy and administrative management responsibilities during this tenure included Chairman, Long-Range Planning Committee, President, Board of Trustees, and President, Oaklawn Health Services, a non-profit health services corporation established to serve community health care needs.

Received a Masters of Public Health (M.P.H.) in Public Health Policy and Administration, 1989, University of Michigan, Ann Arbor, Michigan. March 1990, joined FDA as a veterinary medical officer in the Division of Therapeutic Drugs for Non-Food Animals, Center for Veterinary Medicine. Appointed Director, Office of Animal Care and Use, December 1990.

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November 1998, recipient of a *Certificate of Recognition* under the *Russell & Burch* awards program presented by the Humane Society of the United States.

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Dr. Yu received a B.S. in Biology from Wuhan University, Wuhan, P.R. China in 1982 and a Ph.D. in Chemistry from Wayne State University, Detroit MI in 1988. Since 1993 he has been Assistant Scientist at the Schepens Eye Research Institute of Harvard Medical School
Awards include:
1998: Career Enhancement Award, The Schepens Eye Research Institute
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Errol Zeiger has an M.S. and Ph.D. in microbiology from George Washington University, and a J.D. from North Carolina State University, and is a member of the North Carolina State Bar. He has worked at the National Institute of Environmental Health Sciences, NIH, for 24 years, and has more than more than 30 years experience in biology and toxicology as a laboratory researcher, project director, and scientific program manager. Much of his scientific career has been in the design and direction of laboratory validation studies to determine the effectiveness of short-term genetic toxicity tests, and develop standardized test protocols; the evaluation, interpretation, and integration of toxicological test data; the use of short-term, genetic toxicity tests to predict chronic effects; structure-activity relationships in mutagenicity and carcinogenicity; and the study of mechanisms of chemical mutagenesis. He has lectured on genetic toxicology, test validation, and on scientific publication. Dr. Zeiger was Chair of the Method Validation Workgroup of the federal Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and has been involved with the development and validation of short-term tests. Dr. Zeiger has published extensively in the scientific literature, has been on a number of editorial boards, and is formerly Editor-in-Chief for the journal, *Environmental and Molecular Mutagenesis*. He has recently returned from a 14-month appointment as a consultant with the Environmental Directorate of the Organisation for Economic Co-operation and Development (OECD) in Paris, France, where he wrote and edited health effects Test Guidelines and Guidance Documents.

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Dr. Zendzian is a Senior Pharmacologist in the Health Effects Division of the Office of Pesticide Programs (OPP) of the USEPA. 20+ years of experience in evaluating the toxic effects of pesticides in experimental animals for their potential toxicity to humans. Emphasis on the dermal absorption of pesticides. Developed and wrote the OPP Guideline Subdivision F, Series 85-3 Dermal Absorption of Pesticides.(Now OPPTS Harmonized Test Guidelines # 870.7600 Dermal Penetration)

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Gregor Zlokarnik is Scientific Investigator in the Chemistry Department at Aurora Biosciences Corporation in San Diego, California. His team develops novel fluorescence assays for drug discovery, drug candidate ADME profiling and toxico-genomic applications. These interdisciplinary endeavors

entail the design and synthesis of fluorescent molecular sensors for biochemical and intracellular applications, the study of enzyme kinetics and the creation of mammalian reporter cell lines. He received a B.S. (1984) in chemistry from University of Cologne and a M.S. (1988) in chemistry from Ludwig Maximillians University in Munich, Germany. In 1987, he joined the laboratory of Roger Y. Tsien, then at the University of California at Berkeley, and was awarded a Ph.D. in Physiology in 1994. In 1989, while pursuing his dissertation research project, the laboratory moved to the University of California at San Diego. After a postdoctoral appointment at this institution (1994-96), he became a founding member of Aurora Biosciences Corporation, initially being employed by the company as a Principal Scientist. Dr. Zlokarnik assumed his current position in June of 1999.

Symposium Chairs

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Eugene J. Olajos received his BA and MS degrees from Wayne State University and his Ph.D. from the University of Michigan in Toxicology/Environmental Health Science. Following post-doctoral work at the Massachusetts Institute of Technology and the Albany Medical College, he joined the faculty of the Albany Medical College in the Institute of Comparative and Human Toxicology. He also spent two years on the faculty of Old Dominion University in the Department of Chemical Sciences. He is currently with the Research and Technology Directorate of the Edgewood Chemical Biological Center (ECBC), U.S. Army, Aberdeen Proving Ground, Maryland. Research interests include toxicant-induced neurotransmitter alterations, enzymatic markers as correlates of neuropathology, biochemical mechanisms of neurotoxicity, and *in vitro* alternatives. In addition, he is involved in risk assessment issues concerned with chemical warfare materiel (CWM). He has authored/co-authored over 70 papers, contributed book chapters in the areas of toxicology and genotoxicity, and written reviews covering pesticide toxicology and N-nitroso compounds. Society memberships have included the American Chemical Society, Society of Toxicology, Association of Government Toxicologists, and the International Academy of Environmental Safety. He has served as a reviewer for various toxicology journals.

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Dr. Harry Salem, Chief Scientist for Life Sciences, U.S. Army Edgewood Chemical Biological Center, was previously Chief of the Life Sciences Department, and the Toxicology Division, Chemical Research, Development and Engineering Center, Aberdeen Proving Ground, MD. His research interests and experience are in inhalation and general pharmacology and toxicology. He has served on the editorial boards of several professional journals and is currently a member of the Editorial Board of the Journal of Inhalation Technology, and the Editor-in-Chief of the Journal of Applied Toxicology. Dr. Salem has served as an Associate Professor of Pharmacology at the University of Pennsylvania, Adjunct Professor of Environmental Health at Temple University and is currently Adjunct Professor of Chemical Toxicology at Drexel University.

Dr. Salem was also employed by small and large industry in pharmacology and toxicology. He has served and serves on national and international committees and is chairman of the Technical Committee for the Inhalation Specialty Section of the Society of Toxicology. His professional affiliations include: American Association for the Advancement of Science, American Chemical Society, American College of Toxicology (Charter Member and councilor), American Conference of Governmental Industrial Hygienists, Inc., American Society of Clinical Pharmacology and Therapeutics, American Society for Pharmacology and Experimental Therapeutics, Association of Government Toxicologists where he served as President, Chemical Corps Association Inc., European Society of Toxicology, International Regulatory Pharmacology and Toxicology, International Society on Toxinology, National Capitol Area Chapter Society of Toxicology, where he served as President, Society of Toxicology and Sigma Xi, where he also served as President.

He serves on the Steering Committee for the Society of Comparative Ophthalmology and is a Fellow of the New York Academy of Sciences, the American College of Clinical Pharmacology, the American College of Toxicology, and of the Academy of Toxicological Sciences, where he served on the Professional Standards Evaluation Board, and on the Board of Directors.

In 1989, Dr. Salem was awarded the Decoration for Meritorious Civilian Service for his contributions to the field of toxicology both nationally and internationally.

Dr. Salem is currently a Visiting Professor at Rutgers University. He was recently selected as the Society of Toxicology Congressional Science Fellow for 2001.

He has contributed many scientific papers and is the co-author and co-editor of three volumes of the International Encyclopedia of Pharmacology and Therapeutics on Antitussive Agents, the co-author of a book, "The Biological and Environmental Chemistry of Chromium", the editor of the text, "Inhalation Toxicology" and "Animal Test Alternatives", and co-editor of the book, "New Technologies and Concepts for Reducing Drug Toxicities". Dr. Salem received the B.A. degree (1950) from the University of Western Ontario in Canada, B.Sc. degree (1953) in pharmacy from the University of Michigan, M.A. (1955) and Ph.D. (1958) degrees in pharmacology from the University of Toronto in Canada.

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Dr. Stokes earned his Bachelor of Science degree in biomedical and environmental engineering from the University of Louisville in 1975. He received his Doctor of Veterinary Medicine from the Ohio State University in 1979 and entered active duty in the U.S. Army Veterinary Corps with assignment at the U.S. Army Medical Research Institute of Infectious Diseases at Fort Detrick, where he completed the laboratory animal medicine training program and attained board certification as a Diplomate in the American College of Laboratory Animal Medicine (ACLAM). From 1983-1986 he served as Chief of Veterinary Services for the Department of Clinical Investigation at Tripler Army Medical Center in Honolulu, Hawaii. From 1986-1990 he worked at the National Institute of Child Health and Human Development at NIH, where he directed the Institute's animal care and use program and facilities. In 1990 he moved to the National Institute of Environmental Health Sciences in Research Triangle Park as the Animal Program Director and Chief of the Comparative Medicine Branch. In 1992, he was appointed Associate Director for Animal and Alternative Resources in the Institute's Environmental Toxicology Program, which directs toxicological research and testing for the U.S. National Toxicology Program.

He currently serves as Director of the National Toxicology Program's Center for the Evaluation of Alternative Toxicological Methods, and Co-chairs the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), which coordinates interagency issues related to the development, validation, acceptance, and national and international harmonization of toxicological test methods.

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An Ex Vivo Model and New Parameters for Ocular Irritation Tests

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A simple air lifted corneal organ culture system was used as an ex vivo model for ocular irritancy test. The parameters used to assess epithelial responses included epithelial barrier and paracellular permeability visualized by biotin surface labeling, induced epithelial leakage quantitated by fluorescence retention, and alterations of DNA binding activity of two stress-response transcription factors, NF-kB and AP-1, determined by electrophoretic mobility shift assay. The effects of two surfactants, sodium dodecyl sulfate (SDS) and benzalkonium chloride (BAK), on these parameters and in epithelial cells were studied in cultured corneas. Both SDS and BAK induced tight junction disruption and increased permeability of corneal epithelium in a concentration and time-dependent manner. An increase in DNA-binding activity measured using was observed when cultured corneas were treated with surfactants at concentrations causing minimal to mild ocular irritation, indicating epithelial cell stress-response. Exposure of cultured corneas to SDS or BAK at concentrations causing severe ocular irritancy resulted in a decrease in DNA-binding activity of these transcription factors in epithelial cells. Furthermore, the epithelial barrier function disrupted by surfactants at concentrations causing mild irritation can be recovered in cultured pig corneas. These results indicate that the combination of corneal organ culture and measurements of corneal epithelial permeability and DNA-binding activity of stress-response transcription factors following chemical exposure has the potential to be used as a mechanistically based alternative to in vivo animal testing.

Session Abstracts

Session I: Progress in the Validation and Regulatory Acceptance of Alternatives

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Session I presents an update on the current status of the validation and regulatory acceptance of alternatives to animal testing. Two government organizations, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the European Centre for the Validation of Alternative Methods (ECVAM), are charged with reviewing new toxicological testing methods to determine their validity and promote regulatory acceptance. Each organization has successfully validated new methods that have also received regulatory acceptance by the US Federal Government and the European Union, respectively. Associated with the emergence of these organizations are several issues that need to be addressed. How many new methods will realistically be proposed for formal review, and will ICCVAM and ECVAM have the resources to meet high expectations? What are these organizations doing to identify and prioritize new testing methods to meet regulatory needs? What steps are being taken to harmonize the acceptance of methods that have been validated by both organizations? Although inherent differences exist between the way in which these two organizations operate, the foundation upon which they were founded is similar. That is, as a result of international workshops conducted by both ICCVAM and ECVAM, there is fundamental agreement in the criteria that need to be met for a test method to be considered valid. Clearly, the process for data review is understandably different between the two groups, but the scientific standards upon which the methods are reviewed are consistently high. It is imperative that the US and EU governments bilaterally accept methods validated

by ICCVAM and ECVAM through mutual recognition. Representatives for ICCVAM and ECVAM will discuss the status of their programs and explore these salient issues. Of similar international interest are the recent activities to assess integrated *in vitro* approaches to acute toxicity testing, and the validation and regulatory acceptance of alternative methods to replace the conventional LD 50 test. Progress in these two long-standing issues will be discussed. As a result, Session I provides a comprehensive review on the status of validation and regulatory acceptance of alternatives in the context of the above referenced areas.

Presentation Abstracts

The Interagency Coordinating Committee on Alternative Toxicological Methods and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods: Update on Recent Activities

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Before new test methods are accepted by agencies to support regulatory decision-making, adequate validation is necessary to characterize their usefulness and limitations for specific proposed uses. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) were established by the U.S. government to work with test developers and Federal agencies to facilitate the evaluation and adoption of new test methods. The ICCVAM is composed of representatives from 14 Federal regulatory and research agencies. Test developers are encouraged to interact with ICCVAM/NICEATM throughout test method development, pre-validation, and validation to maximize the likelihood of generating information needed by agencies to adequately assess new test methods. Test method submission guidelines have been prepared by ICCVAM to assist developers in designing validation studies and test method protocols that will adequately address established validation and acceptance criteria. Following validation, ICCVAM and NICEATM coordinate the independent peer review of test methods by international panels of expert scientists. The review process provides for participation by interested parties through public meetings and the opportunity for public comments. Test method peer review reports and ICCVAM recommendations are then provided to Federal agencies where they are considered for regulatory acceptance. Three methods have now been reviewed by ICCVAM independent scientific peer review panels. The first two methods have been accepted by regulatory agencies as recommended by the scientific review panels. These include the murine local lymph node assay, an alternative method for assessing allergic contact dermatitis potential of chemicals, and Corrositex, an in vitro method for assessing the dermal corrosivity potential of chemicals. The third method reviewed is a revised version of the Up-and-Down Procedure (UDP) for assessing the acute oral toxicity of chemicals and products. The UDP is proposed as a substitute for the conventional LD50 test for acute toxicity and uses fewer animals than the conventional method. ICCVAM and NICEATM also recently convened an expert panel to review the validation status of the Frog Embryo Teratogenesis Assay in *Xenopus* (FETAX), a method for assessing the developmental toxicity of chemicals and products. In October, ICCVAM and NICEATM convened an international workshop on in vitro methods for assessing acute systemic toxicity. The expert scientists evaluated the validation status of currently available in vitro methods for estimating acute toxicity, and developed recommendations for research, development, and validation activities that would be helpful in further characterizing and/or improving the usefulness of in vitro methods. The continued development, validation, acceptance, and use of new improved methods are expected to provide enhanced protection of public health and the environment. These methods are also expected to provide increased efficiency and benefit animal welfare by the refinement, reduction, and replacement of animal use.

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The Ocular Surface: Barrier Function and Mechanisms of Injury and Repair at the Ocular Surface

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The cornea being an avascular tissue is dependent on interrelations with adjacent neighbouring tissues and spaces. The components of the cornea, epithelium, endothelium and stroma, are different in structure and function. The epithelium rests on its basement membrane. The cylindric basal cells divide and move to the surface within a few days changing their size, rounding to intermediate cells and flattening to superficial cells, which bear the tear film. The polar structure of the epithelium is characterized by cell migration from the bottom to the apex and sloughing off at the surface. The stroma is a three dimensional network of keratocytes building up the unique extracellular matrix, which forms about 90% of the stromal volume and the firm shelter of the delicate intraocular structures as well as a qualified optical device to project images on the retina. The swelling properties of the cornea, defined by stromal hydration, are important for the stability of the structure and the optical properties. The endothelium controls to a large extent corneal hydration. In the corneal limbus the avascular cornea is connected to the blood system, including its immunological activities. Also, the stem cells of the corneal epithelium are located there.

Severe chemical and thermal injuries of the eyes destroy surface epithelia and cause ischemic necroses of conjunctiva, cornea, sclera, iris, ciliary body, and lids, dependent on the

extent of damage. An inflammatory response follows with leukocyte infiltration and release of inflammatory mediators. Prostaglandins, lipoxygenase products, cytokines, superoxide radicals and lysosomal enzymes are known to be active in eye burn disease. Their activities result in corneal, scleral and conjunctival ulceration, tissue proliferation and scarification, which develop within weeks and months, and may last for years after the accident. Pathophysiological events produce defined clinical pictures. Some agents take special actions, e.g. alkali penetrates within seconds into the anterior chamber, sulfuric acid as well as quick lime burns produce considerable heat. Hydrofluoric acid is highly toxic and induces early necroses. Heat causes deep ischemic necroses and lateron strongly shrinking scars. Onset and intensity of first aid decided on the outcome. Major chemical and thermal injuries need a variety of medical and surgical treatments.

Area and Depth of Injury in a Rabbit Ex Vivo Corneal Model

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The extent of initial corneal injury has been shown by Jester, Maurer and co-workers to be a principal factor determining both early irritation responses and eventual repair in rabbit eyes *in vivo*. Based on this mechanistic understanding of *in vivo* irritation, a method has been developed to measure the area and depth of corneal injury following application of test substances to *ex vivo* rabbit eyes. To develop the *ex vivo* method, rabbit corneas and eyes (obtained from commercial abattoir tissues from animals for human consumption) were exposed on the epithelial surface to four prototype surfactants in preliminary time-course experiments. The corneas were washed, then incubated with fluorescent stains to differentiate live and dead cells and provide clear visualization of corneal cell damage by confocal microscopy. It was found that use of intact eyes for testing (instead of excised corneas) prevented damage occurring from manipulation of excised corneas, more controlled application of test substances, and also prevented photobleaching of the fluorescent stains. A standard test protocol was developed based on the preliminary experiments. The protocol includes a 30-second exposure of the corneas of intact eyes to test substances, a 24-hour post exposure incubation, then measurement of corneal damage by confocal microscopy using differential fluorescent stains (Syto® 10 with Dead Red™). Using these test conditions, validation experiments were conducted with prototype surfactants, acids, alkalies, oxidants and other chemicals characterized previously *in vivo* by confocal microscopy. The *ex vivo* rabbit eye test (ExRET) distinguished chemicals producing slight (epithelium alone), mild/moderate (epithelium and superficial stroma), and severe (epithelium, deeper stroma, possibly endothelial) damage to the corneas, similarly to the *in vivo* responses. It is proposed that the use of *ex vivo* rabbit corneas and quantitative confocal microscopy provides an alternative replacement approach to assess corneal responses that is linked directly to the *in vivo* mechanism of response (area and depth of injury).

Engineered Tissue Equivalents for Ocular Irritancy Testing

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We have developed a prototype tissue engineered human corneal equivalent that comprises cells from the three main corneal layers (epithelium, stroma and endothelium). For a tissue matrix, we used a collagen-chondroitin sulphate-based matrix that was strengthened by crosslinking. To create stromal, epithelial and endothelial layers, respectively, cells were mixed into, and layered below and on top of this substrate. When the epithelium became confluent, the constructs were air-lifted and maintained until used at an air-liquid interphase. The constructs reproduced key physical and physiological functions of human corneas, including cell phenotype, transparency, pump function and gene expression. More recently, we have been able to incorporate an external nerve source that may allow for sensitivity testing that was beyond the scope of the current prototype. Nerve outgrowth from co-cultured ganglia were found

to extend into the cornea through the stroma. Growth cones were found to terminate on overlying epithelial cells. Such engineered corneal equivalents may be in the future be suitable for use as an animal alternative for ocular irritancy testing.

Prevalidation Study Results for the Human Corneal Epithelial Fluorescein Transepithelial Permeability Assay for Eye Irritation, the HCE-T TEP Assay

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Background: The purpose of the HCE-T TEP assay is to provide a biologically and mechanistically relevant alternative to the Draize rabbit eye irritation test for the prediction of the ocular irritation potential of water-soluble chemicals and formulations. Human corneal epithelial cells (the HCE-T cell line) are grown on permeable collagen membranes where they develop into multilayered cell cultures that resemble the structure of the corneal epithelium. The HCE-T cultures develop an epithelial barrier, which is relatively impermeable to fluorescein, like the *in vivo* corneal epithelium. Disruption of the epithelial barrier function in the HCE-T model by a chemical irritant can be measured by its ability to become permeable to sodium fluorescein.

Methods: A Prevalidation Study of the HCE-T TEP assay was conducted to evaluate its performance for the prediction of the ocular irritation potential of water-soluble surfactants and surfactant-based formulations. The Study was designed to provide a preliminary statistical assessment of test method performance and intra- and inter-laboratory assay variability. The Study consisted of the evaluation of 5 test materials by 3 laboratories, with each material tested 4 times in each laboratory for a total of 20 TEP assays per laboratory; 60 TEP assays for the Study. The 5 test materials were surfactant-containing formulations which spanned the range of eye irritation from non-irritating to severe (Draize Maximum Average Score (MAS) 4.8-57.4, and Corneal Score (CS) 0-38). Testing was conducted in a blinded fashion where a third party coded and distributed the test materials. A Prediction Model (PM) developed by statistical modeling of the relationship between the *in vitro* TEP data and the *in vivo* historical Draize MAS test data for 26 water-soluble surfactants and surfactant-containing formulations was used to assess the performance of the HCE-T TEP assay. Subsequently, additional Draize tissue subscores were also used to evaluate the TEP assay results.

Results: The HCE-T TEP assay was successfully transferred from the test method developer (GMEL) to two additional laboratories (IIVS and ECBC). The average TEP assay endpoint for all 5 test materials fell within the 95% confidence intervals of the MAS PM; 55/60 of the individual assays. Sensitivity analysis that included replication variability showed a test method accuracy of 90.0%, a sensitivity of 83.3%, and specificity of 100%. A PM based upon Draize CS was also satisfactorily cross-validated. The average TEP assay endpoint for 4/5 test materials fell within the 95% confidence intervals of the CS PM. Sensitivity analysis showed an accuracy of 93.3%, sensitivity of 88.9%, and specificity of 100%. Chi-square analysis showed significant ($p<0.001$) relationship between irritation classification obtained by the MAS PM or CS PM and classification obtained by the Draize test. The intra- and inter-laboratory variability in TEP assay results were assessed using the variance component analysis and the techniques described by the ASTM Consistency Statistics. These analyses showed no significant differences in both between-laboratory and within-laboratory variability for all 5 test materials. The HCE-T TEP assay is currently being evaluated in a larger Validation Study.

Biographical Sketches

Major General John C. Doesburg

Commanding General
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Major General John C. Doesburg was born in Milwaukee, Wisconsin, in 1947. He is from an Army family and traveled extensively as a child. He attended schools in Pennsylvania, Texas, Germany, Oklahoma, and graduated from high school in Fort Smith, Arkansas. He entered the Army through the ROTC Program at the University of Oklahoma in 1970.

His assignments include: Battery Executive Officer, A Battery, 1st Battalion, 10th Field Artillery; Brigade Chemical Officer, 2nd Brigade, 82nd Airborne Division; Commander, Headquarters Company, 2nd Brigade, 82nd Airborne Division; Commander, 21st Chemical Company, 82nd Airborne Division; Career Program Manager, MILPERCEN; member of the United States Negotiation Team for a Chemical Weapons Treaty, United States Arms Control and Disarmament Agency; Executive Officer, U.S. Army Chemical Activity, Western Command (Johnston Island); Division Chemical Officer, 25th Infantry Division (Light); Commander, 84th Chemical Battalion; Commander, U.S. Army Chemical Activity, Pacific; and Chief, Chemical and NBC Defense Division, Office of the Deputy Chief of Staff for Operations and Plans, Headquarters, Department of the Army, Director, Joint Program Office for Biological Defense.

Major General Doesburg's military education includes the Field Artillery Officer Basic Course, the Chemical Officer Advanced Course, the Command and General Staff College, and the Army War College.

His awards and decorations include the Defense Superior Service Medal, the Legion of Merit with oak leaf cluster, the Defense Meritorious Service Medal, the Army Meritorious Service Medal with five oak leaf clusters, the Army Commendation Medal with oak leaf cluster, the Air Assault Badge, and the Master Parachutist Badge.

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May Griffith is a cell biologist whose expertise is in tissue engineering. Her goal is to develop human-based in vitro biological systems for use as disease models, for toxicology, and ultimately, for transplantation. Her achievements include methods for organ culture of developing bone and corneas, promoting neural differentiation from stem cells, and development of an artificial human cornea. These in vitro systems were developed using cellular and molecular biology techniques. Current goals are to further develop the artificial cornea for use in transplantation and to develop artificial environments to promote neuronal differentiation and re-innervation, particularly within the eye.

James T. MacGregor, Ph.D., D.A.B.T.

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Dr. James T. MacGregor is Director of the Office of Testing and Research (OTR) at the FDA Center for Drug Evaluation and Research. Jim holds a B.S. in chemistry, received a Ph.D. in toxicology from the University of Rochester School of Medicine in 1971, and held a postdoctoral position at the University of California Medical Center, San Francisco from 1970-72. Prior to joining the FDA, he was Director of the Toxicology and Metabolism Laboratory at SRI International (formerly the Stanford Research Institute) in Menlo Park, CA. He has held

academic appointments in toxicology at the University of California, Berkeley and the University of San Francisco. He has been a Diplomate of the American Board of Toxicology since its inception in 1980 and has served on national and international expert toxicology groups and advisory boards for private companies and public research institutions. He has been active in professional societies (including President of the Genetic and Environmental Toxicology Assn. of Northern California, President and Treasurer of the Environmental Mutagen Society, and has served on various committees of the Society of Toxicology). He has served on the editorial boards of Environmental and Molecular Mutagenesis, Mutation Research, and Mutagenesis, and has published more than 200 journal articles, abstracts, and book chapters in the field of toxicology.

Rosemarie Osborne, Ph.D.

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Dr. Rosemarie Osborne is a Senior Scientist in the Human & Environmental Safety Division at The Procter & Gamble Company. Dr. Osborne's responsibilities include the development and use of *in vitro* eye irritation tests and support for eye irritation risk assessments of products and ingredients. Her research has led to the development of tissue equivalent culture methods for eye and skin irritation and corrosion assessments as alternatives to the use of animals. Dr. Osborne has a Ph.D. in Pharmacology from Harvard University and was an NIH Postdoctoral Fellow at the Chemical Industry Institute of Toxicology.

Mr. Michael A. Parker

Deputy to the Commander, SBCCOM
President and Chief Executive Officer of the
SBCCOM Board of Directors

Mr. Parker, as Deputy to the Commander, directs the Army's intensified research, development and acquisition process; readiness for chemical materiel; and technical support to its sister services and to Department of Defense agencies. He also serves as the President and Chief Executive Officer of the SBCCOM Board of Directors.

Mr. Parker was recognized for demonstrating unparalleled leadership in restructuring America's overall chemical and biological defense program. He was instrumental in the formation of the U.S. Army Soldier and Biological Chemical Command to oversee research, development and acquisition of chemical and biological defense equipment and to ensure world-wide commitment to chemical weapons demilitarization and compliance with the 1993 Chemical Warfare Convention. He led a panel of senior leaders dedicated to standardizing chemical and biological defense equipment across the Department of Defense and within NATO. His personal initiative and innovative thinking led to the development of new Joint Service procedures for horizontal integration and coordination of program decisions, and to addressing Congress with a single, proactive voice.

Mr. Parker's efforts culminated in a Joint Service Agreement for unified management of the chemical and biological defense programs of all the Military Departments. The Agreement implements Public Law 103-160 and ensures that the chemical and biological defense mission area is viewed respectfully to counter the proliferation and us or weapons of mass destruction.

In December 1996, he was appointed Program Manager for Assembled Chemical Weapons Assessment, a Congressionally-mandated program examining chemical weapon destruction processes.

Mr. Parker previously served as the Technical Director of the U.S. Army Chemical Research, Development and Engineering Center (now known as the Edgewood Chemical Biological Center). He also served as Acting Project Manager for Binary Munitions and Chemical Munitions and as Science Advisor to the Commanding General, U.S. Army Japan.

A native of St. Louis, Missouri, Mr. Parker holds a Bachelor of Science degree in Mechanical

Engineering from the Missouri School of Mines and Metallurgy. He also attended the University of Michigan and Johns Hopkins University Schools of Engineering. Among his awards and decorations are the Army's highest civilian honorary award, the Decoration for Exceptional Civilian Service, the Army Research and Development Achievement Award, the Army Commander's Medal, the Army Meritorious Civilian Service Award, and the Presidential Rank Award.

Professor Dr. med. Martin Reim

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German Ophthalmologist, emeritus Professor of Ophthalmology and former Director of the University Eye Clinic, of the Faculty of Medicine at the Technical University Aachen (Rheinisch-Westfälische Technische Hochschule - RWTH), Germany.

Born as son of a protestant minister on February 26, 1931 in the Eastern Province of Brandenburg, former Germany, now Poland. Grown up with a brother and two sisters, he attended school there till 1945. Graduated 1951 on a gymnasium in the State of Hessen, Federal Republic of Germany, he studied medicine at the Philipps-University Marburg/Lahn from 1951 - 1957. Two years Internship followed at the University Clinics in Marburg/Lahn: Internal Medicine with Professor H. E. Bock and surgery with Professor W. Zenker. 1958 with Professor G. Schettler promotion to Doctor of Medicine with thesis "On the dietetic significance of unsaturated fatty acids". From 1959 - 1961 research at the Institute of Physiological Chemistry, Philipps-University Marburg/Lahn with Professors Th. Buecher and H. J. Hohorst on the regulation of energy producing metabolism in liver and sceletal muscle. 1961 - 1965 residency in ophthalmology at the Eye Clinic of the Philipps-University Marburg/Lahn. Beside clinical work with Professor W. Straub, research on the energy producing metabolism of the cornea. 1965 - 1966 senior resident, 1966 - 1967 Oberarzt of the Eye Clinic of the Philipps-University Marburg/Lahn.1966 Venia Legendi for Ophthalmology (Privatdozent für Augenheilkunde). Title of the thesis: "Energy producing metabolism and transparency of the cornea." 1967 - 1968 Senior Research Fellow at Retina Foundation and Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, USA. With Professor C. H. Dohlman research on the nutrition of the cornea. Back in Germany, 1968 - 1972 again Oberarzt of the Eye Clinic of the Philipps-University Marburg/Lahn. 1972 - 1973 Provisional Director of the Eye Clinic of the Philipps-University. 1973 - 1996 Full Professor of Ophthalmology and Director of the Eye Clinic, Faculty of Medicine of the Technical University (RWTH) Aachen, Germany. 1985 invited to become Director of the Eye Clinic of the University of Düsseldorf, but 1986 decided to stay at Aachen.

Special academic functions:

Research and ophthalmological activities induced some memberships in scientific societies, such as the Gesellschaft Deutscher Naturforscher und Ärzte (1960), the German Ophthalmological Society (1961), The Association for Research in Vision and Ophthalmology (ARVO) (1968), the Association for Eye Research (AER) (1969), the International Society for Eye Research (ISER) (1977), the German Society for Plastic and Reconstructive Surgery (1980) and The Castroviejo Corneal Society (1982). Also, a number of special academic functions were achieved: 1974 - 1981 Dean for Student Affairs of the Faculty of Medicine. 1981 - 1984 Medical Director of the University Hospital (Klinikum) of the Faculty of Medicine, Technical University (RWTH) Aachen. 1971 - 1981 Regional Representative of the Association for Eye Research (AER). 1981 - 1990 General Secretary of the Association for Eye Research (AER). 1983 - 1988 Member of the Council of the International Society for Eye Research (ISER). 1983 - 1989 Member of the Council and 1985 - 1986 President of the German Ophthalmological Society. 1990 elected Member to the Council of the Societas Ophthalmologica Europaea (S.O.E.), 1993 - 1997 Board of Directors of the Castroviejo Corneal Society, 1994 - 1996 Member of the International Subcommittee of ARVO, and 1994 Organizer of the Cornea Section for the XI. Congress of the International Society for Eye Research (ISER) at New Dehli. 1984 - 1992 elected expert of the Deutsche Forschungsgemeinschaft (DFG), in addition consultant for the Wellcome Trust,

London and the Fonds zur Förderung der wissenschaftlichen Forschung (FWF), Vienna.

Clinical and scientific activities:

Clinical: Corneal and cataract surgery, retinal and vitreous surgery, medical retina, plastic lid surgery, diseases of the ocular surface, eye burns, strabismus - i.e. general medical and surgical ophthalmology.

Research: Since 1961 research was continuously supported by grants from the Deutsche Forschungsgemeinschaft (DFG), Bonn-Bad Godesberg: 1) On metabolism and diseases of the cornea, experimental and clinical eye burns, corneal ulceration, corneal cultures, eye banking. 2) Together with Dr. Ing. Dr. med. Sebastian Wolf, now Professor of Ophthalmology at Leipzig, Germany: Microcirculation of the retina and choroid, first introduction of videofluorescence angiography with fluorescein and indocyanine green in ophthalmology, computerized image evaluation and blood flow measurements. Investigation of diseases of the microcirculation of retina and choroid, age related macula degeneration, diabetic retinopathy.

Publications:

Original and review articles in scientific and ophthalmological journals, some book chapters, and a textbook on ophthalmology, 400 pages, 5 editions, the last one 1996. The textbook was edited on CD-ROM disc 1995. This includes short resumés, the complete long text, extra explanations of figures and interactive questions, extra video animation and the complete index.

Achievements:

1988 together with Professor Dr. med. Christian Teping the Award on Mircrosurgery of the German Ophthalmological Society for the invention of Tenon plasty in anterior segment surgery, especially in severe chemical and thermal injuries.

1989 The Alcon Research Institute Annual Award for merits in corneal research.

1986 The Gullstrand Lecture at Stockholm: "Pathophysiology, surgical and medical treatment of eye burns".

1988 The Louis Emile Javal Lecture at Amsterdam: "Interaction of corneal stroma, epithelium and ocular surface fluid".

Current Status:

Since July 15, 1996 Professor emeritus, retired from clinical and administration responsibilities.

Current research projects :

Together with Privatdozentin Dr. med. Claudia Redbrake: Corneal cultures and eye banking. Together with Privatdozent Dr. med. Norbert Schrage: Development of an artificial cornea for anterior and posterior segment surgery, investigation on the mechanisms of chemical injuries to cornea and conjuntiva, and the influence of rinsing media on the anterior eye segment.

Active membership:

Representative of the German Ophthalmological Society in the European Council of Ophthalmology (S.O.E.). Member of the Jury of the International MSD Chibret Award. Chairman of the German Jury of the Chibret Award. Member of the Jury of the German Ophthalmological Society for dedication of the Leonhard Klein Award for New Achievements in Ophthalmic Microsurgery. Member of the Ethic Committee of the Faculty of Medicine of the Technical University Aachen.

Sherry L. Ward, Ph.D.

Gillette Medical Evaluation Laboratories
Needham, MA
e-mail: sherry_ward@gillette.com

Education:

- B.S. in Biochemistry from Pennsylvania State University, University Park, PA
- Ph.D. in Biochemistry from Michigan State University, East Lansing, MI – thesis on prostaglandin enzymology
- Postdoctoral Fellowship at American Red Cross Holland Laboratory, Rockville, MD – research on protein structure/function of immune system proteins
- Research Fellowship at Johns Hopkins University, Baltimore, MD – research on immune mechanisms in graft vs host disease
- Executive M.S. in Technology Management from University of Maryland

Employed from 1993 to the present by The Gillette Company, Gillette Medical Evaluation Laboratories, In Vitro Research & Testing Laboratory; formerly in Gaithersburg, MD, and presently in Needham, MA. At Gillette Dr. Ward has participated with other scientists in the characterization of the human corneal HCE-T model, and identification and use of biomarkers

for eye irritation using the HCE-T model. Dr. Ward recently led the effort to develop and characterize a human conjunctival epithelial cell line and tissue model. At the present time she is managing a Validation Study of the HCE-T TEP assay to evaluate its ability to predict eye irritation. Dr. Ward also represents the Gillette In Vitro Laboratory with a variety of professional organizations, and works to identify external technologies and collaborations of interest to the group.

Neil L. Wilcox, D.V.M., M.P.H.

Director, Regulatory & Scientific Affairs
Western Hemisphere
The Gillette Company
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Dr. Neil L. Wilcox pursued undergraduate and graduate degrees in veterinary medicine at Michigan State University, East Lansing, Michigan, culminating with a Doctorate in Veterinary Medicine (D.V.M.) in 1971. While practicing clinical veterinary medicine in two Michigan communities, Farmington and Marshall, he was active in several professional and community organizations. In 1989, he received a Masters in Public Health Policy and Administration from the University of Michigan, School of Public Health, Ann Arbor, Michigan.

Dr. Wilcox joined the U.S. Food and Drug Administration, Center for Veterinary Medicine, in 1990, where, as a Veterinary Medical Officer and Director, Office of Animal Care and Use, he represented the Agency on all animal welfare issues. In 1994, he was promoted to Sr. Science Policy Officer, where he represented the Commissioner's Office on a wide range of science policy issues including regulatory acceptance of new toxicological testing methods targeting non-animal biomarkers intended to be more predictive than traditional animal testing models.

In 1999, Dr. Wilcox accepted a position with The Gillette Company as Director, Regulatory and Scientific Affairs, Western Hemisphere. In this capacity, he was challenged with the opportunity to combine regulatory, scientific, and policy management skills to meet corporate responsibilities at the international level.

Appendix B. Monthly Progress Reports Submitted by the Support Contractor

August 2000

Task 60 (Toxicology Symposium):

- In August, we met with the client to plan the symposium. The team developed a draft schedule of sessions, planned social events, and contacted sponsors. We then produced and printed an initial meeting announcement brochure that we mailed to about 400 prospective attendees. We also gathered lists of potential speakers from the session chairs.

- In September, we will finalize arrangements with the anchor hotel for accommodations for the attendees and social functions. We will also continue to coordinate the meeting's daily activities with the National Library of Medicine facility coordinator. After refining the schedule of sessions and the list of speakers, the team will develop a second mailing. We expect to begin processing registrations in September.

September 2000

Task 60 (Toxicology Symposium):

- In September, we arranged a block of rooms at the anchor hotel for participants. Booz·Allen also continued to coordinate the meeting's daily activities with the National Library of Medicine facility coordinator. We began developing a second mailing based on the agenda provided by the session chairs, and then sent official letters of invitation and instructions to the session chairs and steering committee. We began processing registrations for the conference and provided updated agendas to related professional societies for publication in newsletters and posting on internet sites.

- In October, we will obtain the final agenda, print and mail the second meeting announcement, and send letters of invitation to the finalized speakers. We will also finalize the social events for the conference. Following that, we will begin assembling the technical program book based on the final agenda.

October 2000

Task 60 (Toxicology Symposium):

- In October, we finalized the symposium agenda and prepared, printed, and mailed a second meeting announcement. We sent the agenda to the National Library of Medicine facility coordinator. We also provided the updated agenda to our partners who were providing advertising.

- The team continued to process registrations. We received numerous proposals for poster presentations, which we processed and forwarded to the poster session chair. We sent official letters of invitation to the speakers and answered any questions they had about

hotel and travel arrangements. We also ordered meeting supplies, including imprinted pens and folders, for the attendees.

- In November, we will finalize any outstanding food and refreshment issues. We will also prepare the technical program book, which will contain the final agenda and the abstracts and biographical sketches provided by the speakers and poster presenters. We will then travel to Bethesda, Maryland, to provide on-site support to the 28 November conference.

November 2000

Task 60 (Toxicology Symposium):

- In November, we finalized food and refreshment contracts with the hotel and the National Library of Medicine caterer. We finished preparing and printing the *Technical Program Book*, which contains the final agenda and the abstracts and biographical sketches provided by the speakers and poster presenters. We also continued to process registrations and maintained contact with the speakers to answer any questions about their responsibilities. We arranged the necessary audio-visual capabilities with the National Library of Medicine and helped prepare certificates of appreciation for the steering committee and session chairs. In addition, we finalized arrangements for our dinner and keynote speakers.

- We then provided on-site support to the meeting in Bethesda, Maryland, from 27 November to 1 December. We supervised setup of the poster session, processed on-site registrations, and helped the speakers prepare their presentations. We distributed the Program Book, folders, and pens to the attendees. We prepared and printed an addendum to the Program Book containing late information. We also developed and distributed an attendees list.

- In December we will conclude on-site support to the conference and will provide follow-up support. This support will include returning posters to presenters who had to leave the conference early, answering inquiries about travel expenses, and providing instructions to the presenters on how to prepare manuscripts of their presentations for the conference proceedings.

December 2000

Task 60 (Toxicology Symposium):

- In December, we concluded the conference, which boasted over 150 registrants. We finished our on-site support by packing the meeting materials and taking down and returning posters to presenters who had to leave the conference early. We continued to provide instructions to the presenters on how to prepare manuscripts of their presentations for the conference proceedings, collected several manuscripts, and provided them to the client for review.

- In January, we anticipate receiving additional manuscripts for review. We will send letters of appreciation to the speakers and will include further instructions on submitting manuscripts for the proceedings. We will also ensure that catering and other conference charges are properly settled.

January 2001

Task 60 (Toxicology Symposium):

- In January, we continued to receive manuscripts from the conference speakers. We reviewed the manuscripts for format and forwarded them to the ECBC Chief Toxicologist for editing. We also responded to inquiries from speakers about deadlines and format for manuscript submissions. In addition, we ensured that payments were processed to the conference services providers at the Lister Hill Center and Hotel in Alabama.

- In February, we expect to continue receiving manuscripts and corresponding with the speakers and sponsors about the conference proceedings.

February 2001

Task 60 (Toxicology Symposium):

- In February, we continued to correspond with speakers at the conference. We provided information about the format and deadlines for their submission of manuscripts. We also provided the ECBC Chief Toxicologist with copies of the manuscripts for review.

- In March, we expect to continue receiving manuscripts. We will also correspond with the speakers and sponsors about the conference proceedings.

March 2001

Task 60 (Toxicology Symposium):

- In March, this task was modified to add funding which will be disbursed to speakers, compensating them for travel expenses incurred in preparation for and while participating in the symposium. To accomplish this, our team collected expense receipts and vouchers from the speakers and maintained communication with them if further information was needed.

- In April, as soon as the speakers have provided sufficient information regarding their travel expenses, we will process the reimbursements. We will also continue to collect manuscripts from the speakers for the proceedings.

April 2001

Task 60 (Toxicology Symposium):

- In April, our team processed several requests from speakers for reimbursement for their travel expenses to the meeting. We also contacted other speakers from whom we needed additional information in order to process their requests. In addition, we received another manuscript from a speaker to be published in the proceedings.

- In May, we will continue to process reimbursement requests and collect manuscripts as they are submitted.

May 2001

Task 60 (Toxicology Symposium):

- In May, our team continued to process reimbursement requests for speakers' travel expenses to the meeting. We re-contacted speakers from whom we needed additional information to process their requests. We also continued to answer questions from speakers regarding the format for preparation of manuscripts of their presentations for the proceedings.

- In June, we expect this task to be extended at no cost to the government so we may continue to process reimbursements.

June 2001

Task 60 (Toxicology Symposium):

- In June, our team continued to process the remaining reimbursement requests for speakers' travel expenses to the meeting. We also collected two more manuscripts from speakers that will be included in the symposium proceedings.

- In July, we expect to complete processing travel reimbursements. We will also contact the remaining speakers who have not yet submitted manuscripts for the proceedings.

July 2001

Task 60 (Toxicology Symposium):

- In July, we continued to process reimbursement requests for speakers' travel expenses to the meeting. We collected an introductory manuscript and a manuscript of a presentation that will be included in the symposium proceedings.

- In August, we expect to complete processing travel reimbursements. We will also contact the session chairs who will then contact the remaining speakers who have not yet submitted manuscripts for the proceedings.

Appendix C. Preliminary Outline of 2000 Alternatives Proceedings

ALTERNATIVE TOXICOLOGICAL METHODS FOR THE NEW MILLENNIUM

CONTENTS

Engineered Tissue Equivalents for Ocular Injury Irritancy Screening
May Griffith, Ottawa General Hospital

The EpiOcular Prediction Model: A Reproducible In Vitro Means of Assessing Ocular Irritancy
Patrick Hayden, MatTek Laboratories

Cell Biology of Immortalized Human Corneal Epithelial Cells in 3-Dimensional Cultures for In Vitro Toxicology Assays
Roger Beuerman, Doan Nguyen and Bart DeWever, Louisiana State University

Performance of the HCE-T TEP Human Corneal Epithelial Transepithelial Fluorescein Permeability Assay
Sherry Ward, Gillette Company

Regulatory Perspective - Meeting Regulatory Agency Needs for Draize Alternatives
Wiley Chambers, US FDA

Dermal

Alternative Methods for Dermal Toxicity Testing
Robert L. Bronaugh, US FDA

Allergic Contact Hypersensitivity: Mechanisms and Methods
Denise Sailstad, US EPA

Validating In Vitro Dermal Absorption Studies: An Introductory Case Study
Bob Zendzian and Michael Dellarco, US EPA

Utility of gene Array Technology in Skin Biology
Brian Jones, Avon

Keratinocyte-Based Mechanistic Assay for Chemicals That Cause Contact Dermatitis
Anthony Gaspari, University of Rochester

A Molecular Diagnostic Approach to Irritant or Allergic Patch Testing Using DermPatch
Melissa Fitzgerald, Lawrence Rheins, Vera Morhenn, Stacy Humphrey and Nirmala Jayakumar, Derm Tech International

Standardized Human Epidermal and Mucosal Tissue Models for the Screening of Potential Lead Compounds in Pharmaceutical Development
Bart DeWever, Skinethic Laboratories

In Vitro Skin Equivalent Models for Toxicity Testing
Patrick Hayden, MatTek Laboratories

A Case Study in the Use of Alternatives to Determine Mechanism of Action of Sulfur Mustard

Cellular Resistance of Tetrahymena to Sulfur Mustard
Peter Platteborze and William Smith, US Army MRICD

Biochemical changes Induced in human Cells by the Vesicating Agent Sulfur Mustard
William Smith, Offie Clark, Fred Cowan, Maura DeJoseph, Charisse Davenporte and Clark Gross, US Army MRICD

Human Keratinocyte Inflammatory Transcript Gene Activity following Sulfur Mustard
John Schlager, Kashif ali, Hilma Benjamin, Claire Levine, Douglas Avery and James Clark, US Army MRICD

Evaluation of Cytotoxicity Assays of Human Epidermal Keratinocytes Exposed In-vitro to Sulfur Mustard
Susan Kelly, Juanita Guzman, Clark Gross and William Smith, US Army MRICD

Fluorometric Analysis of Intracellular Changes Induced by Sulfur Mustard in Human Cells in Culture
William Smith, Susan Kelly, Offie Clark, Maura DeJoseph, Clark Gross, US Army MRICD

Comparison of Spectrophotometric and Fluorometric Assay of Proteolysis in Cultured Human Cells Exposed to Sulfur Mustard
Susan Kelly, Charlene Corun, Marian Nelson, Clark Gross and William Smith, US Army MRICD

Effects of Low Dose Sulfur Mustard on Growth and DNA Damage in Human Cells in Culture
William Smith, Bethany Toliver, Offie Clark, Janet Moser, Eric Neally, Juanita Guzman and Clark Gross, US Army MRICD

Imaging Sulfur Mustard lesions in Basal Cells and Human Epidermal Tissues by Conical and Multi-Photon Laser Scanning Microscopy
Robert Werrlein and J.S. Madren-Whalley, US Army MRICD

Suppression of Sulfur mustard-Increased IL-8 in Human Keratinocyte Cell Cultures by Serine protease Inhibitors: Implications for Toxicity and Medical Countermeasures
Fred Cowan, Clarence Broomfield and William Smith, US Army MRICD

Therapeutic Approaches to Dermatotoxicity by Sulfur Mustard
William Smith, Robert Cassillas, Clark Gross and Irving Koplovitz, US Army MRICD

Integration of Sulfur Mustard Data Derived from Alternative Toxicology Methods to Define Mechanism of Action
William Smith, US Army MRICD

Neurotoxicology (Molecular Biomarkers, Transgenics and Imaging Techniques)

Overview
William Slikker, NCTR/FDA and Thomas Sobotka, CFSAN/FDA

Molecular Neurotoxicology of 6-Hydroxydopamine and Methamphetamine: Lessons Perused from Transgenic Models
Jean Lud Cadet, US NIDA

Development and Evaluation of In-Vitro Imaging Techniques Used to Screen Agents That Affect Neuronal Differentiation
Stanley Barone, US EPA

Potential Applications of Noninvasive Imaging in Toxicological Research
David Lester, FDA

A Microassay Method Using a Neuroblastoma Cell Line to Examine Neurotoxicity of Organophosphate Mixtures
Laurie Roszell, B.C. Kramer and Glen Leach, US Army CHPPM

Molecular Strategies for Neurotoxicity Screening: Moving Beyond the One Compound, One Mechanism Approach
James O'Callaghan, CDC

Priority Setting and Framework for Endocrine Disruptor Screening
G.E. Timm, J.F. Darr, C.J. Flessner, P.W. Kennedy, A.F. Maciorowski, T.R. O'Bryen and J.D. Walker, US EPA

Development of a Model of Integrin Expression as a Molecular Biomarker for Early Sensitive Detection of Neurotoxicity
Joyce Royland, US EPA

Role of Transgenics and Toxicogenomics in the Development of Alternative Toxicity Tests

Overview
Jerry Heindel, NIEHS

The Application of Genomics and Proteomics to Toxicological Sciences
Stephen Chevalier, William Pennie and Ian Kimber, Zeneca Central Toxicology Laboratory

Use of Transgenics in Carcinogenicity Testing: An Update
Denise Robinson, ILSI

Toxicogenomics and Toxicity Testing
Ben Van Houten, NIEHS

Changes in Gene Expression After Exposure to Organophosphorus (OP) Agents
Sekowski, J.W., O'Connell, K.P., Khan, A.S., Valdes, J.J., Vahey, M., Nau, M., Khalil, M. and Eldefrawi, M.S., ECBC, Walter Reed AIR, and University of Maryland

Mammalian Gene-Trapping with the Beta-Lactamase Receptor: Application to Toxicogenomics
Gregor Zlokarnik, Aurora Biosciences Corporation

Noninvasive Real-time Toxicodynamics
Karen Steinmetz, Xenogen

Role of Imaging in the Development of Alternative Toxicity Tests

Nanostructures and Toxicology
Martin Philbert, University of Michigan

MRI and Biophotonic Imaging
Brian Ross, University of Michigan

Two Photon Microscopy: Application to Toxicology
Peter So, MIT

Recent Innovations in Alternatives

Archival Data in Toxicology: Minimizing Need for Animal Experiments
Millard Mershon, SAIC

Archival Data and Information Management at the Library of Congress: An Overview with Special Reference to Biomedicine and Toxicology
Roman Worobec, Library of Congress

In Silico Cardiac Toxicity: Speeding the Discovery of Therapeutics Through High Performance Computing
C.K. Zoltani and S.I. Baskin, U.S. Army Research Lab, and U.S. Army Medical Institute of Chemical Defense

Submillimeter-Wave Frequency Studies of the Vibrational Modes of Deoxyribonucleic Acid: A Metric for Mutagenicity?
T.R. Globus, B.L. Gelmont, M. Bykhovskaia. J. Hesler, D.L. Woolard, A.C. Samuels, J.O. Jensen, H. Salem and W.R. Loerop, U of VA, US Army Research Office and US Army ECBC

Trophoblast Toxicity Assay (TTA): A Gestational Toxicity Test Utilizing human placental Trophoblasts
Harvey Kliman, Yale University